Summer 2007

Fate of the vegetal yolky cells in the direct developing frog Eleutherodactylus coqui

Uma Karadge

Fate of the vegetal yolky cells in the direct developing frog *Eleutherodactylus coqui*

A thesis

Presented to the Bayer School of Natural and Environmental Sciences

Department of Biological Sciences

Duquesne University

In partial fulfillment of the requirements

For the degree of Master of Science

By

Uma B. Karadge

Thesis Advisor: Richard P. Elinson, Ph.D.

Thesis Committee:
John A. Pollock, Ph.D.
Sarah Woodley, Ph.D.

Name: Uma B. Karadge

Thesis Title: Fate of the vegetal yolky cells in the direct developing frog *Eleutherodactylus coqui*.

Degree: Masters of Science

Date:

Approved: _____
Dr. Phil Auron, Chair
Department of Biological Sciences

Approved: _____
Dr. Richard Elinson, Advisor
Department of Biological Sciences

Approved: _____
Dr. John Pollock, Committee member
Department of Biological Sciences

Approved: _____
Dr. Sarah Woodley, Committee member
Department of Biological Sciences

Approved: _____
Dr. David W. Seybert, Dean
Bayer School of Natural and Environmental Sciences

ABSTRACT

The Puerto Rican tree frog, *Eleutherodactylus coqui,* is a striking example of a deviation from the indirect mode of development among amphibians. *E. coqui* does not have an intermediate tadpole stage. It hatches out directly as a free-living froglet, deriving nutrition from the yolky material attached to its intestine. The innermost of the three germ layers, the endoderm, forms the lining of the gut. In the amphibian model *Xenopus laevis*, the endoderm originates from the vegetal hemisphere. The cells, which are originally present on the vegetal surface of the embryo, end up forming the definitive gut. They, thus, provide nutrition as well as contribute towards the definitive gut. Using fate mapping, I determined the fate of the vegetal yolky cells in *E. coqui*. When one of the vegetal yolky cells in *E. coqui* is injected with a lineage tracer, Fluorescein Dextran Amine (FDA), the label ended up only in the yolky endoderm attached to the intestine in the developing embryo. There is no label in any part of the gut or body in all stages of development. The yolky endoderm is gradually used up by the developing embryo and the label disappears completely after all of the yolky endoderm is used. I conclude that the vegetal yolky cells in *E. coqui* are only nutritional in function. When the yolky endoderm was being utilized, the label appeared in the mesonephric kidneys. This suggests that when the yolk was being used, FDA was picked up by blood and cleared during filtration in kidneys. FDA, which has a molecular weight of 10,000, freely passes the glomerular filtration membrane and enters the nephric tubules. Confocal microscopy resolved that the dye is trapped in the form of aggregates or vesicles in the individual epithelial cells lining the nephric tubules. Some of the FDA entering the tubules is likely cleared in the form of dull fluorescent debris in the cloaca of the frogs. I also carried out fate mapping of the cells situated towards the animal pole (above-AV) and the cells near the equator (below-AV) and found that cells from both of these areas ended up in the definitive gut. They also contributed towards tissues and organs of mesodermal and ectodermal origin. Taking together all these data, I conclude that the vegetal yolky cells in *E. coqui* provide nutrition only. The gut is derived from cells closer to the animal pole. A more comprehensive fate map and further analysis of the utilization of yolk in *E. coqui* is required to better understand dye clearance and labeling in the mesonephric kidneys.

ACKNOWLEDGEMENTS

In this effort of working for my Masters, am indebted to my parents for their constant support and inspiration. They have given me some of the best lessons in life and held my hand in both hope and despair. To them, I dedicate this thesis. I am very grateful to my brother for his unconditional support and encouragement; you are the best brother in the world! Thanks to my sister for always giving a push ahead and being my best friend. One person who deserves special thanks is my husband, Srikanth. His love and support truly helped me get through.

I express my deep sense of gratitude to my advisor Dr. Elinson. You are the best advisor, I could have wished for. So profound is your knowledge and the spell of your teaching and your way of working, that I am fascinated by developmental biology now. I am sorry for my habit of starting sentences with ‘but’ and ‘however’. This thesis is a result of the great amount of work I put you to do! Thanks for also taking care of coqui embryos while I was away home.

Among my lab members, Kim deserves special thanks for helping me and answering my stupid questions. Thanks to Seung Yun, Michelle, Pete, Oscar, Steve, Zach and Kelly. It was fun working with you guys. I would also like to thank my friends, Khadija, Puneet, Shalini and Sudhir.

I wish to thank Dr. Pollock and Dr. Woodley for serving as my committee members. Dr. Pollock, am very grateful for your confocal lessons and sparing time for my questions from your busy schedule.

Thanks Pam and Judy, it was a pleasure to walk to the office. Thanks very much to Mary Ann from Deans Office for her help and ‘fighting’ for me during some payment issues.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Direct development involves the development of an embryo to an adult without an intervening and free living tadpole or larval stage. An extreme example of this is the Puerto Rican tree frog *Eleutherodactylus coqui*. Unlike other anuran amphibians, *E. coqui* does not metamorphose into an adult frog. Instead it hatches out as a free living froglet. The yolky material attached to its intestine provides nutrition for its development. Fertilization is internal and a clutch of about forty eggs is laid on land. The eggs are incubated by the father for about three weeks after which tiny froglets hatch out of the jelly capsules. Each *E. coqui* egg is twenty times the volume of a *Xenopus laevis* egg.

I. Endoderm and gut formation in *X. laevis*

The amphibian embryo divides and redivides to form three germ layers- ectoderm, mesoderm and endoderm. Ectoderm gives rise to the epidermis and nervous system, while mesoderm forms blood, heart, muscle, skeleton and kidneys. Endoderm, the innermost germ layer, forms the lining of pharynx, esophagus, stomach and intestines, in short the lining of gut. Endoderm also forms the epithelium of liver, gall bladder, pancreas and the respiratory system (Dale and Slack, 1987). The smooth muscle and connective tissue of the gut comes from the mesoderm (Chalmers and Slack, 1998). Extensive studies on the formation, specification, differentiation and the interaction of germ layers during gut formation are done in the amphibian model *X. laevis* (Horb and Slack, 2001).

In *X. laevis*, the endoderm, originates from the vegetal hemisphere (Dale and Slack, 1987). The vegetal region undergoes complicated morphogenetic events during gastrulation to create a tubular primitive gut, the archenteron. The cells which are originally present on the vegetal surface are internalized to line the floor of archenteric cavity. The cells which are on the animal surface line the blastopore lip of the gastrula and end up as the archenteric roof. The archenteric cavity closes and is later replaced by a definitive lumen of the gut (Chalmers and Slack, 2000).

Chalmers and Slack (2000) investigated the morphogenetic movements associated with gut formation in *X. laevis*. Using the lipophilic dye DiI, they specifically labeled the large yolky cells on the floor of the archenteron, and using FDA, they labeled the cells in the middle of the ventral endoderm. The middle of the ventral endoderm lies in between the floor of the archenteron and the endoderm, which lies next to the mesoderm. Both groups of cells, the yolky cells and the middle of the ventral endoderm cells, were incorporated into the intestinal epithelium and so are not digested during development. These double labeling experiments showed that both types of cells are incorporated into the intestinal epithelium by a process of radial intercalation. DiI labeling indicated that the floor endoderm intercalates with the dorsal endoderm and the middle endoderm intercalates with the ventral or lateral mesoderm. Thus, the endoderm fate map of Chalmers and Slack showed that all regions of the endoderm give rise to a part of the gut or respiratory epithelium. There is no indication of any type of cell loss or death by way of apoptosis.

II. Endoderm and gut development in *E. coqui*:

Direct development in *E. coqui* is said to be derived because its ancestors had a tadpole stage. Biphasic development in anurans consisting of an intervening tadpole or larval stage is considered to be a primitive condition (Townsend and Stewart, 1985; Elinson, 1990; Callery *et al*., 2001). Evolution of direct development has occurred independently as many as 10 times during the evolution of anurans (Fang and Elinson, 1996). *E. coqui* represents an extreme case of the above. Traces of tadpole stage are found in the *E. coqui* embryo in the form of tail, rudimentary gills and operculum (Callery and Elinson, 2000a). *E. coqui* has many deviations from the biphasic development of anurans because of the large yolk-rich vegetal cells to support direct development. This has shifted *E. coqui* towards a trend of adoption of terrestrial life. It is surprising though, that it undergoes thyroid hormone-dependent metamorphosis, which occurs before hatching (Callery and Elinson, 2000b).

The evolution of the amniote egg consisted of an increase in the amount of yolk and hence increase in the size of egg. A large amount of yolk is required to support the development of an organism, which directly forms a free living adult. In *X. laevis* and *Rana*, the animal cytoplasm constitutes roughly 50% of the egg volume whereas this region occupies only 3% of the egg volume in *E. coqui*. This increase in the vegetal cytoplasm has probably lead to the expansion of the nutritive endoderm in *E. coqui*. Expansion of the nutritive endoderm is a major evolutionary change in the transition of development between anamniote amphibians and amniote reptiles and birds. The large

vegetal yolk mass is secondarily covered by the expansion of body wall in *E. coqui*. This expansion is analogous to the spreading of the chorion to enclose the yolk in chicks (Callery *et al*., 2001).

In *E. coqui*, the elimination of the tadpole stage is coincident with the presence of a large yolk filled egg. An *E. coqui* egg measures 3.5 mm in diameter compared to the small 1.3 mm *Xenopus* egg. Cleavage divisions produce a clump of small animal cells but only slowly divide the large vegetal yolk mass (Fang *et al*., 2000, Ninomiya *et al*., 2001). In *E. coqui*, the fourth cleavage division, which forms the sixteen cell embryo, is the first horizontal cleavage division. This is in contrast to *X. laevis* where the third cleavage division, which forms the 8 celled *Xenopus* embryo, is the first horizontal cleavage division (Elinson and Beckham, 2002). The small 8 animal cells in *E. coqui* represent roughly 1% volume of the embryo (Ninomiya *et al*, 2001). Despite their small size and the relative volume they occupy, these animal blastomeres contribute to the ectoderm as well as the mesoderm. At this stage, the 8 large vegetal yolky cells are incompletely divided. The cleavage furrows extend very slowly due to the hindrance of large amount of yolk.

Since *E. coqui* embryos are not pigmented, it is not possible to recognize the prospective ventral and dorsal sides until early gastrula (NF stage10, Nieuwkoop & Faber, 1956). At NF10, the dorsal lip of the blastopore forms on the prospective dorsal side. At this time, the blastocoel roof is very thin and semi-transparent. Fate mapping of the blastocoel roof at NF10 using vital dyes showed that early gastrulation in *E. coqui* is similar to that of *X. laevis* embryo (Elinson & Fang, 1998). One third of the roof on the dorsal side contributes to the head ectoderm whereas the rest of the blastocoel roof forms

the ectoderm over the yolk. This ectoderm present over the yolk actually stretches around the embryo to cover the large yolk mass. The original ectoderm over the yolk mass apoptoses and is replaced by the body wall. This coverage is unique among amphibians (Elinson and Fang, 1998; Elinson and Beckham, 2002).

III. Molecular control of endoderm specification in frogs

The body plan and the primary germ layers in *X. laevis* are established by two localized maternal determinants. These are a dorsal determinant and a mesoderm/endoderm determinant. While dorsal determination is a signaling pathway that involves the signaling molecule β- catenin, mesoderm/endoderm specification involves a transcription factor, VegT (Kimelman *et al*., 1992; Harland & Gerhart, 1997; Heasman, 1997). VegT is a T-box transcription factor, and its RNA is synthesized during oogenesis. It becomes localized to the vegetal hemispheres in stage IV oocytes and early *X. laevis* embryos (Zhang & King, 1996; Horb & Thomsen, 1997; Stennard *et al*., 1996). Loss of function studies in *X. laevis* showed that VegT is a maternal regulator and initiator of a complex zygotic molecular network specifying the endoderm (Xanthos *et al*, 2001). Maternal VegT directly initiates the transcription of *Bix1, Bix4* and other homeobox genes (Casey *et al*, 1999; Tada *et al*., 1998) and the nodal gene *Xnr1* (Kofron *et al*., 1999). Other downstream targets of VegT include homeodomain proteins of the Mix/ Bix/ Mixer family, the zinc finger factors *GATA-4, GATA-5 and GATA-6*, and the HMG domain transcription factors, *XSox17α and XSox17β.* (Hudson *et al*., 1997; Henry and Melton 1998; Xanthos *et al*., 2001; Clements *et al*., 2003). VegT depleted embryos do not express the endodermal genes like *endodermin*, *XSox17, Xlhbox 8*, *insulin* and

IFABP, implying its role in endoderm formation (Zhang *et al.*, 1998; Xanthos *et al.*, 2001). VegT is absolutely essential for the differentiation of the embryonic endoderm. Larvae developing from VegT depleted oocytes totally lack endoderm organs (Xanthos *et al.*, 2001).

The mesoderm inducing signals, belonging to the transforming growth factor TGFβ family and the nodal related genes, require VegT for initiation of their expression. Derriere and the nodal related genes, *xnr*-1, -2, -4, -5 and –6 are involved in this signaling (Clements *et al.*, 1999; Kofron *et al.*, 1999; White *et al.*, 2002). *Xnrs* and the TGFBβ signals are essential for mesoderm induction and are required for the maintenance of endodermal gene expression (Yasuo and Lemaire, 1999; Engleka *et al.*, 2001; Clements and Woodland, 2003).

XSox17 is an HMG box transcription factor expressed in cells fated to form endoderm. It is expressed throughout the vegetal region, which forms the vegetal region in *X. laevis* (Hudson *et al.*, 1997). The two isoforms of *Sox 17*, *XSox17α* and *XSox17β* in *X. laevis* are both necessary and sufficient for endodermal development (Hudson *et al.*, 1997). Northern Blot analysis in *X. laevis* demonstrates that the two transcripts *Xsox17α* and *XSox17β* are expressed at late blastula; there is no maternal expression. *In situ* hybridization experiments revealed the expression of *XSox17* to be superficial in the form of a ring around the blastopore lip as well as internally throughout the presumptive endoderm (Hudson *et al.*, 1997). Whole mount *in situs* on bisected gastrulas at NF11 confirm the endodermal expression of *XSox17* (Sinner *et al.*, 2004). Inhibition of endogenous *XSox17* with a dominant negative *XSox17* construct resulted in abnormal gut development at low concentrations and gastrulation blockage at higher concentrations

(Hudson *et al*., 1997). Inhibition of both *XSox17α* and *XSox17β* lead to an affected early gut phenotype wherein midgut, proctodeum, stomach and liver are affected to varying levels (Clements *et al*., 2003).

In *E. coqui*, a major part of the yolk laden vegetal mass lacks *EcVegT* RNA (Beckham *et al*., 2003) and mesoderm inducing activity (Ninomiya *et al*., 2001). Radioactive RT PCR and *in situ* hybridization demonstrated the presence of *EcVegT* and *EcVg1* RNAs predominantly near the animal pole of *E. coqui* oocyte (Beckham *et al*., 2003). *EcVegT* and *EcVg1* are the *E. coqui* orthologues of *VegT* and *Vg1* respectively. *Vg1* RNA is localized to the vegetal cortex and encodes a TGF-β family member, which is suggested to be involved in dorsal mesoderm formation and left-right asymmetry (Melton 1987; Weeks and Melton, 1987; Thomsen and Melton, 1993;Kessler and Melton, 1995; Hyatt and Yost, 1998). RT PCR showed that the animal blastomeres, which are formed from the first horizontal cleavage division, inherit half of the *EcVegT* and *EcVg1* transcripts. These animal cells represent only 1% of the embryo, which gives an idea of the very little involvement of the rest of the 99% of the embryo in endoderm and mesoderm formation. *EcVegT* and *EcVg1* RNAs are found in the animal hemispheres of fully grown oocytes as well as during oogenesis. This is the reason why half of EcVegT and EcVg1 transcripts are restricted to the eight animal blastomeres resulting from the first cleavage division (Beckham *et al*., 2003). In *E. coqui*, the blastopore lip arises equatorially and much closer to the animal pole as compared to *X. laevis*. Mesoderm inducing activity and probably TGFβ signaling is located equatorially and sub-equatorially in the early gastrula and is restricted to the superficial cells (Ninomiya *et al*., 2001).

EcSox17, the orthologue of *XSox17* was cloned recently (Sean Williamson, GenBank EF 397004).). *In situ* hybridization experiments showed that similar to *XSox17, EcSox17* was expressed in the form of a ring around the blastopore lip, and expression extended towards the anterior in the bisected embryo (Singamsetty, 2005). The ventral lip was only superficially stained whereas the yolk-rich vegetal region at the yolk plug did not show any *EcSox17* expression in gastrula. Expression around the blastoporal lip suggests that this region contributes to most of the definitive endoderm. Although the vegetal yolky cells failed to show any staining in *in situ* experiments, *EcSox17* expression was revealed by RT PCR. The relative roles of *EcSox17* expression at the blastoporal lip and in the yolk-rich vegetal region are unknown.

IV. Hypothesis

Animal expression of *EcVegT* suggests that endoderm arises more animally in *E. coqui* and that the yolk-rich vegetal region does not contribute to the definitive endoderm. At the time of hatching, the developing gut in *E. coqui* consists of very narrow anterior and posterior gut tubes. These tubes are attached to a large yolk-rich central region with a lumen (Langer, 2003). Proliferation assays recognized comparatively heavy proliferation in the anterior and posterior regions of the yolk-rich tissue. Thus, the anterior and posterior tubes may represent definitive gut tissue. As mentioned before, the blastopore lip is formed more towards the animal pole, it is very unlikely that the vegetal yolky cells will differentiate to form the definitive gut. They may function as a nutritive tissue just like the uncleaved yolk of chick eggs. Thus, it can be hypothesized that the vegetal yolky cells in *E. coqui* do not form definitive gut tissue but serve only a nutritive function.

Here, I propose to examine the fate of these yolky vegetal cells in *E. coqui*. In *E. coqui*, gut development can be hypothesized to occur in two ways (Fig. 1). According to Hypothesis 1 (Fig.1b), the definitive gut epithelium might differentiate initially in a small area and then spread on both sides until it fuses to form a complete intestinal lining with a lumen. In order to achieve this, a large number of yolky cells must apoptose, during the development and elongation of the gut and be replaced by the spreading intestinal epithelium. According to the second hypothesis (Fig. 1c), the yolky cells adjoining the differentiated endoderm might be transformed into intestinal epithelial cells. This process continues until a complete lining of intestinal epithelium is formed surrounding the lumen of the gut.

V. Approach:

In the first step of fate mapping, FDA will be injected into one of the large vegetal blastomeres of the fertilized *E. coqui* egg. The injected eggs will be allowed to develop until TS 15 (TS= Townsend and Stewart 1985 stages), which takes approximately three weeks. The guts of the frogs developed from the injected embryos will be dissected to detect the presence or absence of labeled cells. In order to find out the relative contributions of other cells, two other types of injections will be done. One will involve injecting FDA into the cells close to the animal pole and the other will involve injecting FDA into the cells above but close to the equatorial midline of the 60-cell *E. coqui* embryo. Since the cells near the equator have inducing activity, these cells might form some parts of the gut. Contribution of cells near the equator can be visualized by the presence of fluorescence in the gut tube. If the first hypothesis (Fig.1b) holds true, then

labeling a vegetal blastomere will not yield any fluorescence in the gut. If gut development occurs as per the second hypothesis (Fig.1c), then progeny of the labeled vegetal blastomere will end up in the definitive gut.

MATERIALS AND METHODS

I. Collection and staging of *E. coqui* embryos

E. coqui embryos used in experiments were obtained from naturally mating frogs as described previously (Elinson *et al*, 1990). These frogs were wild caught in Puerto Rico under the permits issued by the Departmento de Recursos Naturales and in Hawaii under Injurious Wildlife Exports Permits issued by the Department of Land and Natural resources. They were maintained in the laboratory as per rules provided by IUCAC. Fertilized embryos were collected and placed on a filter paper moistened with 20% (0.2X) Steinberg's solution in plastic Petri dishes. Steinberg's solution was prepared from two 20X stock solutions- Steinberg's A and Steinberg's B. Stock A contains 1.16 M NaCl, 13mM KCl, 17mM $MgSO_4.7H_2O$ and 6.7mM Ca $(NO)_3$, and Stock B contains 100mM Tris adjusted to pH 7.4 with 1N HCl. The embryos were raised in Petri dishes and checked daily for their developmental progress. The developing embryos were staged according to Townsend and Stewart (1985). Townsend and Stewart classified the embryos into fifteen stages from the uncleaved egg (TS1) until hatching (TS15).

II. Dejellying

The embryos to be dejellied were flooded with 20% (0.2X) Steinberg's in a plastic Petri dish. This allows the jelly layers around the embryos to swell, facilitating their removal. At about the 60-cell stage, the outer and middle jelly layers were removed manually using two very fine forceps. The dejellied embryos were then left submerged in 20% Steinberg's. At this point, they were stained with 0.0025% Neutral Red in 20%

Steinberg's for 30 min in a plastic Petri dish. Staining of the embryos made it easy to distinguish the animal and vegetal poles and also to count the number of blastomeres in an otherwise nonpigmented background of coqui embryos. This step was eliminated later, and unstained embryos were directly injected because the stained embryos had a relatively lower survival rate.

III. Microinjections

Embryos to be injected were placed on a plastic mesh, which was mounted, in a small plastic Petri dish with the help of petrolatum. The plastic mesh had squares with a size of 2 X 2 mm wherein coqui embryos could be placed. Normal, healthy looking embryos at approximately the 60-cell stage were selected, and the embryos on the plastic mesh were partially submerged in 5% Ficoll prepared in 100%(1X) Steinberg's. Glass needles were made using a needle puller (KOPF Needle/Pipette puller, Model 730). They were trimmed with forceps to get rid of the extra long tip and to get a desired inner diameter. The needles were beveled with a micropipette grinder (Narishige, Model EG-44) at an angle of 45^0 to get a sharp tip. A needle with a larger diameter pierces more than one blastomere and causes excessive leaking of the embryo contents whereas a very small diameter needle bends and does not pierce the blastomere appropriately. FDA (Fluorescein Dextran Amine, 10,000 MW, anionic, lysine fixable, Molecular Probes) was prepared by dissolving FDA in deionized water at 50 mg/ml. This solution was dispensed into eppendorf tubes in 25 µl aliquots and stored at -20^0C. Prior to the injections, the tubes were thawed on ice and centrifuged at 13.4 X 1000 g for 5 min. A needle was first filled completely with mineral oil avoiding any bubbles. A single drop (1 µl) of FDA was

placed on a small strip of Parafilm and drawn through suction into the oil filled glass needle with Drummond "Nanoject Variable" Automatic Injector (Cat. No. 3-000-203-XV). Observing under a dissecting microscope, the embryos were rotated using a hair loop to get the desired blastomere on top. The blastomere was injected with 9.2 nl of FDA in the form of a single shot. Injected embryos were left for 2 to 3 min in the plastic mesh and then transferred with a plastic dropper to a flat bottom plastic well plate.

IV. Culturing injected embryos

Each injected embryo was placed in a single well of the flat bottom plastic well plate containing 5% Ficoll in 100% Steinberg's and gentamycin. The dimensions of each well are 1cm diameter by1.5 cm depth. Gentamycin 1000 X stock was made by dissolving 50 mg of gentamycin in 1 ml of deionized (dI) water. This stock was added to the well plates at the rate of 1µl per ml of 5% Ficoll. This 9-well plastic well plate was kept in a large Petri dish, and the injected embryos were left in the above solution overnight. This solution was discarded the next morning and replaced with 20% Steinberg's. The embryos were submerged partially in 20% Steinberg's, which was changed regularly and the embryos were observed daily for developmental progress. The injected embryos were allowed to develop as long as they survived. After hatching, the young frogs were removed from the plastic well plate and transferred by gentle tapping to a Petri dish containing 20% Steinberg's. The level of 20% Steinberg's was kept low since *E. coqui* frogs are terrestrial and tend to jump and sit on the walls or the ceiling of Petri dish. Again the solution in the Petri dish was changed daily.

V. Dissection

Any freshly dead frogs, developing from injected embryos, were dissected with a pair of sharp forceps to open the ventral part of the body in such a way that most of the internal organs are exposed. Such dissected frogs were fixed in MEMFA in small glass vials and stored at 4 ^{0}C. Frogs at the desired stage or that were sick were anesthetized with 1% MS 222 (3-Aminobenzoic Acid Ethyl Ester, Methanesulfonate salt, SIGMA) in 100% Steinberg's solution for 4-5 seconds, dissected in 200% (2X) Steinberg's and then fixed in MEMFA. MEMFA contains 100mM MOPS, 0.2mM EGTA, 1mM $MgSO_4$ and 3.7% formaldehyde at pH 7.4. It was prepared fresh every time using 1 part 10X MEM Stock Solution (1M MOPS, 2mM EGTA, 10mM $MgSO_4$ autoclaved), 1 part 37% formaldehyde stock solution and eight parts dI water. The fixed frogs were dissected further to separate different parts of the body, e.g. foregut, hindgut, liver, mesonephros, heart, pronephros (if present), vertebrae, spinal cord, brain, eyes, forelimbs and hindlimbs. Each separated part or organ of the frog body was mounted in a drop of anti-fade buffered glycerol on a glass slide. The anti-fade buffer was prepared using 25 mg n-propyl gallate in 5 ml PBS. Five ml glycerol was added to the above mixture, and the pH was adjusted to 8.0-8.6 using 1 N NaOH. The mounted organs were covered with a cover glass, which was gently pressed down, and the slides were kept at 4^{0}C. For controls, frogs, developing from uninjected embryos, were anesthetized at the desired stage, dissected in 200% (2X) Steinberg's and fixed in MEMFA. They were dissected and mounted on slides as above.

VI. DAPI staining

DAPI stock was prepared by mixing 1 mg DAPI in 1ml dI water and stored at 4^0C in a glass vial covered with aluminum foil. For DAPI staining of debris in the cloaca, the cloaca of uninjected *E. coqui* frogs at two week post-hatched stage was punctured with a sharp forcep. The debris that oozed out was collected on a slide, dried and washed three times with 1X Phosphate Buffered Saline (PBS). A10X stock of PBS was made by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH2PO4 in 800 ml of dI water. The pH was adjusted to 7.4 using HCl; final volume was made up to 1000 ml with dI water, and the solution was autoclaved. The slide was then covered with 3.7% paraformaldehyde in 1X PBS for 10 min to fix the material. The paraformaldehyde solution was prepared by dissolving 3.7 g paraformaldehyde in 100 ml 1X PBS with intermittent shaking and incubating at 60^0C in a water bath. Paraformaldehyde was aspirated off the slide and the slide was again washed with 1X PBS three times. After getting rid of all PBS on the slide, DAPI (1 µl DAPI stock mixed with 1ml 1X PBS in an eppendorf tube) was added. The slide was covered with a Petri dish and left undisturbed for ten min. The slide was washed three times with 1X PBS to get rid of DAPI. After the final wash, a few drops of fresh 1 X PBS were placed on the slide, and the specimen was covered with cover glass.

VII. Imaging

The specimens were examined using a Nikon Microphot-SA microscope with fluorescence filters for FITC. Confocal imaging was done using Leica Confocal Microscope (Leica Microsystems). The wavelength was set at FITC WIDE and special

FITC GREY, and the lasers used were 488 Ar/ArKr set at minimum. Images obtained from both microscopes were processed using Adobe Photoshop.

RESULTS

I. Fate Mapping

Fate mapping is a technique in which a specific blastomere is injected with a lineage tracer like FDA (Fluorescein Dextran Amine), RDA (Rhodamine Dextran Amine) or GFP (Green Fluorescent protein). Then the development of that blastomere is traced in order to know its destiny in the developed embryo. An *E. coqui* embryo at 40-60-cell stage looks different from a *Xenopus* embryo at this stage (Fig. 2). Three cell types in a cleaving *E. coqui* embryo can be readily distinguished by virtue of their different sizes. The animal pole of the embryo has two to three tiers of actively dividing small cells, followed by comparatively larger cells. The vegetal pole has very big, incompletely divided, yolk filled cells.

The animal vegetal boundary (AV) is an imaginary transient line, which separates the animalmost small cells (Above-AV blastomeres) from the relatively larger below-AV blastomeres. Above-AV FDA injections were done on a single blastomere immediately above the AV while the below-AV FDA injections were done on a blastomere immediately below AV boundary. Vegetal blastomere FDA injections targeted a single large vegetal blastomere. Large amount of yolk in these vegetal blastomeres provides a hindrance for their complete division. Since these are incompletely divided, the dye is expected to end up in more then one cell. The developed frogs were scored for results according to their respective TS stages until hatching at TS15. Frogs that hatched and then survived for a week after the day of hatching (Day zero to day six) were grouped as newly hatched frogs. The ones that survived beyond day six until day thirteen were

grouped as one week post-hatched, beyond day thirteen until day twenty as two weeks post-hatched, and beyond day twenty seven as four weeks post-hatched. Table 1 summarizes the number of embryos scored for all three types of injections at various stages.

II. Vegetal Pole injections

When one of the vegetal blastomeres was labeled with FDA in an embryo at 40-60 cell stage, the label ended up only in the large yolky endoderm and not in the differentiated gut tissue (Fig. 3). This was true in 43 out of the 49 developing frogs examined at various stages coming from various clutches. The remaining of the six frogs had tiny specks of label in liver; however, they did not have any dye in the gut tube. Frogs at TS11 – TS15 have a large number of yolk-rich cells in the developing gut. Both of the TS11 embryos examined had all their yolky endoderm labeled. The developing gut tube – the foregut and the hindgut did not have any label. All four TS13 embryos coming from the same clutch also had their entire yolky endoderm labeled (Fig. 3a). Similar results were obtained in all five TS15 embryos (Fig. 3b) and in all thirteen newly hatched froglets (Fig. 3c). Very bright fluorescent green streaks occasionally appeared in the yolky endoderm of both TS15 and newly hatched frogs (Fig. 3e).

The one and two week post-hatched frogs have used up considerable amounts of yolk and are left with a small lump of yolk-rich cells in a relatively thicker gut tube. Such frogs showed distinct staining in the yolk-rich cells only (Fig. 3d, 4a). There was no dye in any part of the gut except for very tiny specks of dye in the liver in two out of thirteen newly hatched and four out of fifteen one week post-hatched frogs. There was no case of

labeling in the gut tube or intestine in any of the fifty frogs analyzed. The utilization and disappearance of yolky endoderm in these embryos corresponded with the appearance of fluorescence in the cloaca (Fig. 4b, c). This fluorescence was strikingly different from the cellular appearance of the labeled yolky endoderm and first appeared in a weak form towards the end of one week post-hatched stage. The intensity of fluorescence was maximum in the cloaca in two weeks post-hatched frogs. Thus the intensity of labeling in the cloaca corresponded inversely with the amount of yolky endoderm. The fewer the number of yolk-rich cells left in the developing gut, the greater the fluorescence in the cloaca.

The cloaca however did not show labeling in any of the TS9, TS11, TS13, and TS15 embryos. Only one out of six newly hatched froglets showed fluorescence in the cloaca. Fluorescence was not seen in the one four week post-hatched frog. Upon puncture of the cloaca, it was observed that the fluorescence in the cloaca was because of a thick liquid that oozed out. This oozed debris also occurred in the control frogs at the same stage and neither autofluoresced nor had intact nuclei as revealed by DAPI staining (Fig. 4d, e)

A few very late two weeks post-hatched frogs (19-20 days after hatching,) did not have any fluorescence in the cloaca. These frogs had very little to negligible amount of yolky endoderm left behind, which could be visualized in the form of a few fluorescent green specks (Fig. 6a, b). The only frog that survived until the four weeks post-hatched stage had no yolk-rich cells left and absolutely no label at all in any part of the gut.

Apart from yolky endoderm, the only organs labeled in the entire body of the frogs were the mesonephros or pronephros depending upon the stages at which the frogs were scored. Very young frogs with a copious amount of yolky endoderm showed distinct labeling in pronephros. One out of two TS9 frogs had label in the pronephros, but both the TS11 embryos did not. Pronephros was stained in all four TS13 embryos (Fig. 5a) but only in two out of the five TS15 embryos. Frogs after TS15 showed no pronephros labeling corresponding with the eventual disappearance and nonfunctionality of these structures. Disappearance of dye in pronephros was coupled with the gradual appearance of label in mesonephros. There was no label in the TS9 (n=2) and TS11 embryos (n=2), but label started appearing weakly as early as TS13 (n=4). Two out of five TS15 embryos from three different clutches had a weak labeling in mesonephros (Fig. 4b). The fluorescent signal became gradually stronger and distinct from newly hatched stage onwards (Fig. 5 c). Twelve out of thirteen newly hatched and all the one week post-hatched (n=15), two weeks post-hatched (n=8) and the four week post-hatched frogs (n=1) had label in mesonephros. It was not clear whether the label was inherent to the mesonephric tissue. It was also possible that the dye was accumulating in the tubules if it was being cleared by blood after the utilization of the labeled yolky endoderm. Confocal imaging of mesonephros did not resolve whether the dye was in the lumen of tubules or in the cytoplasm of cells (Fig. 5e, f).

III. Above-AV injections

FDA labeling of a blastomere above the animal vegetal boundary (AV) showed that cells in this region contribute to the formation of organs of ectodermal, mesodermal and endodermal origins (Table 2). These cells are originally situated above the imaginary AV which separates comparatively smaller and actively dividing animal blastomeres from larger, slow dividing yolk filled vegetal blastomeres.

Ectoderm: The above-AV blastomere contributed to yolky endoderm and the organs of ectodermal origin like epidermis of eye (Fig. 7a), brain, forelimb and hindlimb skin (Fig. 7d) and the spinal cord (Fig. 7b, c).

Mesoderm: Mesodermal contribution of the cells above-AV was seen in pronephros and mesonephros (Fig. 7e, f), muscles of forelimbs and hindlimbs, vertebrae (Fig. 8b) and occasionally in bones (Fig. 8a).

Endoderm: These cells also contributed to the endoderm giving rise to some parts of the liver and the definitive gut. The foregut, hindgut and stomach showed labeling (Fig. 8c, d). Labeling in the yolky endoderm was minimal and was usually restricted towards the edges. Relative contributions of above-AV injections are summarized in Table 3.

In a few above-AV injected embryos, the label ended up only in the forelimbs/hindlimbs and not in the endoderm/liver. There were few others, which had the exact opposite condition (Table 3). Above-AV contributions occasionally ended up in the formation of either side of many organs or only one out of the two paired organs, like eyes and limbs. In six out of fifteen embryos, only one of the eyes was labeled. This condition was often found when one side of the brain and the spinal cord were labeled. In

four cases, labeling in only one eye was coupled with labeling of the forelimb and hind limb on that side.

Frogs developed from above-AV injections also had a label in pronephros and mesonephros. Both these structures were labeled early in development, i.e. in TS6 –TS7 in pronephros and TS9 in mesonephros in contrast to the late appearance of label in vegetal pole injections. No frogs were scored at a stage earlier than TS6-7 in either vegetal pole or above-AV injections. In case of vegetal pole injections, label appeared in pronephros in TS13 and in mesonephros in the newly hatched stage. Thus label appeared first in TS9 in above-AV injections, since a TS6-7 embryo did not have dye in mesonephros. The pattern of label in mesonephros with the utilization of yolk and loss of label was similar to vegetal pole injections. Confocal imaging did not reveal any difference in the labeling pattern between above-AV and vegetal pole injected pronephros and mesonephros.

Embryos with above-AV injections have a very low survival rate as compared to those with vegetal pole injections. None of the frogs survived after one week post hatched stage. Therefore it was not possible to detect whether labeled debris appeared in the cloaca after the yolk was gone.

IV. Below-AV injections

The blastomere below-AV also contributed to all the three germ layers. Labeling occurred in the same organs as above-AV contributions but to varying degrees (Table 3). There were maximum contributions in yolky endoderm, mesonephros and foregut and

minimal in eyes, limbs, skeleton and the hindgut (Fig. 9a, b). The extent of dye in the yolky endoderm was intermediate between above-AV and VP injections (Fig. 9d). Similar to above-AV injections, there were a few below-AV injection cases in which only half of some organs and only one of the two paired organs were labeled. Except for two embryos (one TS15 and one TS13) mesonephros was stained in all below-AV injections. Confocal imaging revealed that the staining pattern in below-AV injected frog mesonephros was exactly the same as above-AV and VP labeled mesonephros. Below-AV injections could not be scored for the presence of labeled debris in cloaca, since no embryos survived past one week post-hatched stage. However one of the frogs from one week post-hatched stage did have a label in cloaca.

DISCUSSION

I. Principal Findings

Fate mapping of the vegetal yolky cells in *E. coqui* showed that they do not form the definitive gut. These cells do not contribute to any other tissue of the frog body. With the utilization of yolky endoderm, however, the mesonephros became labeled. The cloaca also became fluorescent from debris that accumulated after most of the yolky endoderm was utilized. The definitive gut is derived from above-AV and below-AV cells. These cells also contributed to tissues of mesodermal and ectodermal origin.

II. Vegetal yolky cells

Labeling of the vegetal cells resulted in labeling the entire yolky endoderm only. There was no case of staining in gut tube or any other tissue of the body. This finding shows that the first hypothesis (fig. 1b) of gut formation in *E. coqui* holds true. The vegetal yolky cells are labeled but are completely lost and do not appear in the definitive gut. Since the yolky cells are large in size as compared to the animal cells and some of them are incompletely divided, labeling only one of these resulted in labeling the entire yolky endoderm. This yolky endoderm is attached to the intestine and is used by the developing embryo. The older frogs, which survive beyond TS15, have used up a considerable amount of these yolk-rich cells. Frogs, which have used up all the yolky endoderm, did not show any trace of FDA in the gut tube. When the yolky endoderm was starting to get utilized, dye appeared in pronephros and mesonephros as well.

III. *E. coqui* gut is derived from cells closer to the animal pole

When cells above-AV and below-AV were lineage traced with FDA, their progeny ended up in the definitive gut and other tissues in the body. The yolky endoderm was also labeled to varying extents. The below-AV cells made more contributions to the foregut and hindgut. Below-AV cells contributed 50-90% of the time towards the formation of foregut as compared to the above-AV cells, which contributed only 10-50% of the time. Similarly, the below-AV cells contributed 10-50% of the time towards hindgut whereas the above-AV cells did so less than 10% of the time. Apart from the gut, the above-AV and below-AV cells were involved in the formation of organs of ectodermal and mesodermal origin. Major ectodermal and mesodermal contributions of the above-AV and below-AV cells were in the brain, spinal cord, eyes, vertebrae and mesonephros. In conclusion, the above-AV cells contributed to the ectoderm most of the time whereas below-AV cells gave rise to the endoderm more frequently. Thus, the above-AV and below-AV cells form the definitive gut in *E. coqui*.

The staining patterns in the brain and spinal cord resulting from above-AV injections were interesting. Only one of the halves of the entire brain and spinal cord was stained. This condition was often coupled with labeling of only one of the two paired organs like forelimbs, hindlimbs and eyes. This bilateral labeling pattern is because the injections targeted a single cell, which gave rise to only one half of that particular organ. Cells giving rise to the other half of the brain or spinal cord should be present on the opposite side of the embryo.

Above-AV and below-AV injections also labeled the yolky endoderm attached to the intestine; however, they had a distinct pattern. Since the above-AV cells were far from the large vegetal yolky cells, a small fraction of their progeny ended up only in the dorsal, peripheral region of the yolky endoderm. The below-AV cells are closer to the vegetal yolky cell, which is why their progeny ended up occupying comparatively larger areas of the yolky endoderm.

IV. Molecular basis of gut formation and endoderm development *in E. coqui* vs. *X. laevis*

The finding that vegetal yolky cells do not form the definitive gut is consistent with the fact that *EcVegT* and *EcVg1*, the *E. coqui* orthologues of *VegT and Vg1* respectively, are expressed predominantly towards the animal pole of *E. coqui* oocytes and not in the vegetal cortex (Beckham *et al.*, 2003). In *X. laevis*, a dorsal determinant and a mesoderm / endoderm determinant establish the body plan and the primary germ layers (Yuge *et al*, 1990; Holowacz and Elinson, 1993, Marikawa and Elinson, 1997). The mesoderm / endoderm determinant involves the functioning of a T-box transcription factor, *VegT* (Heasman *et al.*, 1989; Kimelman *et al.*, 1992; Harland and Gerhart, 1997; Heasman, 1997). *VegT* is synthesized during oogenesis in stage I oocytes and becomes localized to the vegetal cortex in stage IV oocytes (Zhang & King, 1996;Horb & Thomsen, 1997; Stennard *et al.*, 1996). *VegT* functions as a maternal regulator and initiates a zygotic molecular network, which specifies the endoderm. It is responsible for the initiation of Bix genes (Casey *et al.*, 1999; Tada *et al.*, 1998), *Xnr* (Kofron *et al.*, 1999), important zinc finger transcription factors like GATA and the HMG domain transcription factors *Xsox17α* and *Xsox17β,* all important in specifying the endoderm

(Hudson *et al*., 1997; Henry & Melton, 1998; Xanthos *et al*., 2001; Clements *et al*., 2003). Unlike *Xenopus VegT, EcVegT* is expressed far less in the vegetal hemisphere of *E. coqui* embryos. It is expressed away from the vegetal hemisphere and more towards the animal pole (Beckham *et al*., 2003). Thus, it is unlikely that the vegetal cells in *E. coqui* have any ability to specify the endoderm.

Similarly, *Vg1* RNA, involved in the dorsal mesoderm formation (Kessler and Melton, 1995) and left-right asymmetry (Melton, 1987; Weeks & Melton, 1987, Thomsen & Melton, 1993; Kessler & Melton, 1995; Hyatt & Yost, 1998) is again localized to the vegetal hemisphere. In *E. coqui*, half of the *EcVg1* transcripts are restricted to the 8 animal blastomeres, which are formed as a result of the first horizontal cleavage division. This suggests that the mesoderm inducing activity and the *VegT* expression and consequently endoderm inducing activity is shifted more animally in *E. coqui* as compared to *X. laevis* (Ninomiya *et al*, 2001; Beckham *et al*., 2003).

VegT induces *Sox17* expression in *X. laevis*, which is important in specifying the endoderm (Hudson *et al*., 1997; Engleka *et al*., 2001). *EcSox 17* is expressed from cleavage until hatching in *E. coqui* (Singamsetty, 2005*). EcSox 17* continues to be expressed in post-hatching stages suggesting its involvement in endoderm specification. This is likely because gut development is not complete at hatching in *E. coqui*. Although pancreas, liver and other endodermal organs are formed, there is a lot of yolky endoderm attached to the midgut. *In situ* hybridization for *EcSox17* shows that it is expressed only on the marginal zone near the blastopore lip at gastrula. This suggests that the definitive gut is likely to be largely derived from the cells in this area. There is no ventral expression of *EcSox17* in the area of yolk plug. Although *in situ* hybridization for

EcSox17 suggests that the vegetal yolk-rich region does not form the definitive endoderm, RT PCR experiments have a contradictory story. RT PCR experiments show that the vegetal yolk-rich region does have *EcSox 17* RNA along with the dorsal and vegetal halves (Singamsetty 2005). The same vegetal region is devoid of *VegT* (Beckham *et al.*, 2003).

V. Gut development in *X. laevis* vs. *E. coqui*

In *X. laevis* embryos, the vegetal region undergoes complex morphogenetic events during gastrulation to produce the archenteron, the tubular primitive gut. Cells from the vegetal surface of the early gastrula form the floor of the archenteron, and cells on the equatorial surface end up forming the roof of the archenteron. The archenteric cavity later closes and is replaced by the definitive gut lumen (Chalmers & Slack, 2000). At NF stage 14, Chalmers and Slack labeled the yolky cells lying in between the floor of the archenteron and the roof of the archenteron with DiI and found that these yolky cells were incorporated into the gut at NF stage 45. These yolky cells do not disintegrate during development but become an integral part of the gut in *X. laevis*. Thus, all the gut and respiratory epithelia comes from endoderm, which in turn originates from the vegetal hemisphere in *X. laevis*. Cells in this vegetal hemisphere have large amounts of yolk but still they get incorporated into the gut, meaning they provide nutrition as well as form the endoderm.

In contrast, the vegetal yolky cells in *E. coqui* provide nutrition only and completely disappear from the definitive gut. The definitive intestinal epithelium does not come from the vegetal region in *E. coqui*.

VI. Mesonephros and debris in cloaca

Besides yolky endoderm, the pronephros and mesonephros were also labeled by the vegetal pole injections. The label in pronephric tissue was found only from TS9 to TS15. Disappearance of dye in pronephros after TS15 was probably because this structure stops functioning and may be lost once the mesonephros develops and becomes fully functional (Lee, 2005). While the dye was starting to disappear from pronephros, mesonephros was getting labeled during TS14-TS15. The label in mesonephros appeared weakly before TS15 but became stronger after the newly hatched stage. This pattern of labeling starts after the yolky cells start being utilized, since the frogs showed a gradual increase in the strength and amount of label while the yolky endoderm was gradually disappearing. The vegetal yolky cells thus contributed to the pronephros and mesonephros.

While the yolk was being utilized, drab green fluorescence appeared in the cloaca. This fluorescence was particularly interesting because it persisted in the cloaca for a very specific period of time in development. The fluorescent signal was strong in two weeks post- hatched frogs, because of debris in the cloaca. Histological studies (Buchholz, unpublished) of gut development in *E. coqui* showed that during gut development, the yolky endoderm was being utilized, but the big yolky cells immediately adjacent to differentiated intestinal epithelial cells were dumping their cell contents in the lumen of the gut. Such yolky cells appeared like big empty cells.

Yolky cell contents in the lumen had intact nuclei. There is a possibility that these contents came down the hindgut and imparted a drab fluorescence to the cloaca. Cloacal debris however did not have intact nuclei. The debris was more like a ground substance and was eliminated as waste.

These observations suggest that while the embryo was developing, the yolk was being used up, but the dye was being cleared by blood. When blood carrying FDA reaches the mesonephros for purification, FDA gets trapped in the mesonephric tubules. The debris was cleared from the hindgut, but label in mesonephros was permanent and persisted until the frogs died. Confocal imaging of whole mount mesonephros or sections showed that the dye was trapped in the mesonephric tubules in the form of aggregates or vesicles within the tubule cells. The unlabelled, dark center is the nucleus as determined by DAPI. Small amounts of FDA may be excreted through the mesonephros along with urea, which appeared, later in the debris in the cloaca.

The fluorescent debris in the cloaca has another plausible explanation. It is possible that *E. coqui* frogs are using the yolk rich cells directly as food instead of absorbing the yolk through specialized cells around the yolk sac as in chick. TUNEL staining of the developing gut did not show any apoptosis of the yolk rich cells on a large scale (Buchholz, unpublished). It is possible that very few cells might be apoptosing at a given time. If the yolk is directly being used as food, then the developing gut should be functional and lined with well developed intestinal epithelial cells. In an otherwise developed and fully functional gut, the food is digested with different secreted enzymes and then absorbed by the columnar intestinal epithelial cells in order to be incorporated

into the blood. It is essential to determine whether the developing gut tube has this ability to digest and absorb food.

If the yolky endoderm was labeled, although to a varying extent in all the three types of injections, then the dye should also appear in the cloaca in frogs developing from above-AV and below-AV injections. This could not be detected because the greatest drawback of above-AV and below-AV injections is low survival rate. Almost all frogs died before they reached a stage where the cloaca becomes filled with debris. There was only one case of a below-AV injection, when the frog survived long enough to show this result.

The frogs developing from above-AV and below-AV cell injections also had a label in the pronephros and mesonephros, but label in both the organs appeared earlier than that in vegetal pole injections- TS6 –7 in pronephros and TS9 onwards in mesonephros. Since the frogs were not scored for labeling before TS6-7, the possibility of presence of dye before these stages in pronephros and mesonephros cannot be ruled out. The yolky endoderm was also labeled in above-AV and below-AV injections. The extent of labeling in yolky endoderm was far less compared to the vegetal pole injections. As interpreted above, if the dye were present in the yolky endoderm, then it should appear in the pronephros and mesonephros as it is used up. It actually did in both above –AV and below-AV cases, but at a stage earlier than vegetal pole injections. It is possible that the dye was inherent to the pronephric and mesonephric tissue since above-AV and below-AV cells contributed to mesodermal organs. The pattern of labeling in both above-AV and below-AV injections, however, was similar to mesonephric labeling in vegetal pole injections. Thus, it is of particular interest and importance to know where exactly the

dye in mesonephros was coming from, and whether it is really inherent to the mesonephric tissues as opposed to the appearance of dye in the later stages in case of vegetal pole injections as a result of clearance by blood.

VII. Filtration in the kidney

Since the vegetal pole, above-AV and below-AV injections resulted in labeling the mesonephros, it is important to consider some aspects of filtration in the kidneys. In amphibians, the pronephros is the embryonic kidney formed early in development. Mesonephros is the final and functional adult kidney. The nephron is the functional unit of kidneys and each kidney is made up of a very large number of nephrons. A nephron is divisible into three parts: the glomus, the proximal and distal tubules and the duct. The glomus filters blood and empties the filtrate into a cavity known as coelom. The filtrate from the coelom is collected by ciliated tubules and passed on to the proximal tubules. Proximal tubules function in solute resorption and waste excretion. The distal tubules carry urine, which passes down the mesonephric duct to the cloaca (Duellman and Trueb, 1986).

An amphibian nephron can be divided into four parts - a glomerulus, a proximal convoluted tubule, a distal tubule and a collecting duct (Fig. 10). During urine production, blood is filtered through glomerular capillaries. The filtered fluid or glomerular filtrate enters the coelom. Glomerular filtrate is actually a virtual ultrafiltrate of the blood plasma (Karnovsky, 1979). Glomerular capillaries consist of a filtration membrane made up of endothelial cells and podocytes. The endothelial cells have pores or fenestrations and the podocytes have filtration slits, which allow the passage of

molecules from blood. Water, glucose, sodium, chloride, vitamins, amino acids, urea, ammonia and small plasma proteins pass the filtration membrane and are released into the coelom in the form of glomerular filtrate. Large proteins and blood cells are not filtered through the filtration membrane; they stay in blood. Glomerular filtrate in the coelom then enters the proximal tubule. Resorption of valuable constituents of blood, like glucose, chloride and sodium ions, from the glomerular filtrate occurs in this region. The tubule cells in the proximal tubule region absorb the useful solutes, and these are returned to the blood, which flows through peritubular capillaries. The proximal tubule region is also capable of transporting wastes and foreign particles from blood to the tubular urine. Resorption of water from tubular urine takes place in the region intermediate to the proximal and distal tubules.

The exact function of the distal tubule is not known, but it is believed to aid in adjusting the acidity of urine. Substances not resorbed by the tubules appear in the urine. Urine is passed on from the distal tubule to the mesonephric duct. All ducts collectively empty into the cloaca in amphibians and excreted out of the body (Duellman and Trueb, 1986).

The glomerulus acts as a primary barrier for filtration of substances from blood. Plasma proteins, high molecular weight proteins and other protein aggregates do not pass through the glomerular filtration membrane. They therefore never enter the coelom and are never excreted. With proteins, the restriction for filtration through the glomerular filtration membrane begins at a molecular weight of 14,000 and increases with increase in molecular weight up to 68,000 (Renkin & Robinson, 1974, Bott & Richards, 1941). Albumin, which has a molecular weight near 60,000, is filtered in very small amounts

through the glomerulus. It enters the coelom and the tubules, but is subsequently resorbed by tubule cells and returned back to blood (Bott & Richards1941; Berggard 1970). The protein Equine cyt C with a molecular weight of 12,000 is extensively filtered through glomerulus and is cleared through urine (Karnovsky and Ainsworth, 1972).

Dextrans have been used as tracers to determine the permeability barrier of the glomerular filtration apparatus. Dextrans behaved like proteins as far as glomerular filtration is concerned. Dextrans of molecular weight 55,000-60,000 Da were not filtered through the glomerulus in mammalian kidneys (Wallenius, 1954; Mogensen, 1968; Arturson *et al.*, 1971; Hardwicke *et al.*, 1972). Extremely tiny amounts of dextrans in blood belonging to the molecular weight range of 55,000-60,000 Da managed to pass the glomerular filtration membrane barrier and appeared in the glomerular filtrate. Dextrans of molecular weight 32,000 intravenously infused into rats were extensively filtered through the glomerulus. Very high concentrations of this dextran appeared in the urinary spaces and were later cleared through urine. As expected dextrans of molecular weight 125,000 were retained in the blood. Dextrans that passed the glomerular filtration apparatus were found in the lumen of all segments of nephron. In the proximal tubules, the dextrans were found in the apical vacuoles where they apparently undergo resorption (Caulfield and Farquhar, 1974). Dextrans filtered through the glomerulus are reported to be resorbed by the tubules (James and Ashworth, 1961).

Filtration of substances through the glomerulus is also dependent on the Einstein –Stokes radius (ESR) of the molecules. Of the common tracers used to study glomerular filtration, like Equine cyt C (12,000 Mr, ESR- 15 Å), horseradish peroxidase (40,000 Mr, ESR- 30 Å) and ferritin (480,000 Mr, ESR- 61 Å), Equine cyt C has no restriction for

glomerular filtration. A small molecule, PPD- 49609 (14,500 Mr, ESR – 20 Å) from tubercle bacillus showed very high filtration through glomerulus and also passed through urine in the amphibian *Necturus* (Bott and Richards, 1941). This suggests that, molecules with an ESR around 20 Å freely pass the glomerular filtration membrane. Molecules with an ESR above 30 Å, like high molecular weight dextrans, myeloperoxidase and catalase are restricted to a certain extent by the glomerulus (Graham and Karnovsky, 1966; Venkatachalam *et al*., 1970). Creatinine and inulin have been extensively used to measure glomerular filtration rate (GFR) of mammalian and amphibian kidneys, since they are completely cleared from blood after a certain period of time and excreted through urine. Creatinine (113 Mr) and inulin (5000- 5,500 Mr) have molecular weights well below the cut off points for glomerular filtration (14,000 and above for proteins, around 50,000-60,000 for dextrans). Renkin and Gilmore (1973) noted that mammalian and amphibian glomeruli behave similarly in their filtering properties and their filtration barriers in both have similar theoretical pore sizes.

VIII. FDA presence in *E. coqui* mesonephros

In case of *E. coqui*, when the labeled yolky endoderm starts getting utilized, the FDA is likely picked up by blood. FDA-laden blood then comes to the kidneys for filtration where FDA passes the glomerular filtration membrane and enters the tubules. FDA molecules can be in the lumen of the tubules or resorbed by the tubule cells and stored in their vesicles. Substances resorbed by tubule cells should eventually be returned to blood via peritubular capillaries. This may not be the case with FDA in the tubule cells, because FDA is not found in blood. It is possible that tiny amounts of FDA are

secreted back in the tubules and excreted along with urea, and they appear in the form of dull fluorescent debris in the cloaca. All the FDA in blood may not be cleared at once. It would be interesting to note if fluorescent debris appears continually in the cloaca if the frogs survive long enough. In this way, trapped FDA might be cleared. FDA is found in a dispersed form in kidneys, but there is a possibility of it being aggregated upon fixation (Caulfield and Farquhar, 1944). Since FDA exists in a dispersed form, there is a possibility of it being resorbed and passed back into circulation. There are, however, no reports on resorption of FDA or dextrans back into circulation.

The onset of glomerular filtration was demonstrated in a teleost pronephros by injecting small, fluorescent tracers directly into circulation of developing embryos (Drummond *et al.*, 1998). In zebrafish, the pronephros is well developed with an infolded glomerular filtration apparatus at 40 hours post fertilization. When rhodamine dextran amine (RDA, 10,000 Mr) was injected in the circulatory system of zebrafish embryos, it was not filtered through the glomerular membrane and did not enter the pronephric duct, suggesting that the opening of the coelom is closed at this time. In 48 hours post fertilization embryos; however, RDA entered the lumen of the pronephric duct. In 56 hours post fertilization embryos, RDA was present in lumen, and it also entered pinocytic vesicles of epithelial cells lining the tubules and duct. The dye did not accumulate in the lumen of the gut or any other structure of the body (Drummond *et al*, 1998).

A similar approach of injecting a fluorescent tracer into the heart can be used to determine the onset glomerular filtration in *E. coqui* pronephros. The pronephros can be excised and its staining pattern can be compared to labeled pronephros found in frogs that

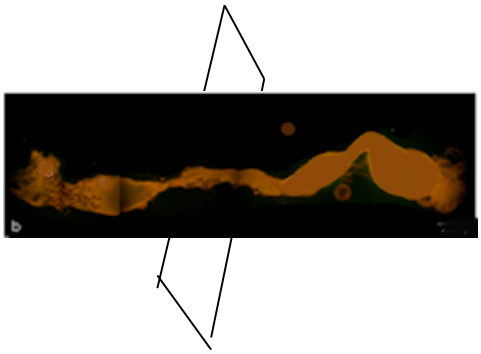
developed from FDA injections. Such injections would also detect whether FDA is cleared from blood and trapped in the mesonephros.

IX. A bigger picture

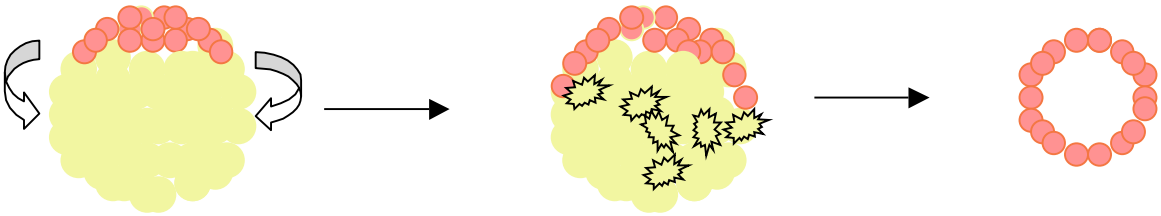
Large amounts of yolk in the vegetal cells of *E. coqui* early embryos decrease the surface area and volume of the region occupying the animal cytoplasm and expand the prospective endoderm. This was the biggest change that occurred to support direct development. Although *E. coqui* has a big egg and large amounts of yolk, it differs from birds and reptiles. The chick egg also has only a little animal cytoplasm, occupying the superficial animal region of the embryo. Most of the embryo/egg is comprised of yolk. Cleavage in chick is meroblastic, however, and the yolk is undivided and not internalized. In *E. coqui*, the yolk cleaves slowly but does so entirely and gets internalized by gastrulation and by secondary coverage of the body wall. The present fate mapping study shows that these yolk rich vegetal cells contribute neither to any of the organs of endodermal origin, nor to any of the body tissues. The yolky cells are indeed nutritive in function.

Fig. 1: Two possible ways of gut development in *E. coqui*. (a) Developing gut of *E. coqui* at TS15. Circular diagrams in (b) and (c) represent transverse sections of the gut as shown in (a). (b) Endoderm differentiates initially in a small area to form intestinal epithelial cells. It spreads on both sides until it fuses to form a complete intestinal epithelial lining of a hollow gut. To get rid of the large number of yolky cells, they must apoptose.(c). The yolky cells adjacent to newly differentiated intestinal epithelium are transformed to intestinal epithelial cells. The process continues until a lining of epithelium is formed in the gut. Few yolky cells must apoptose.

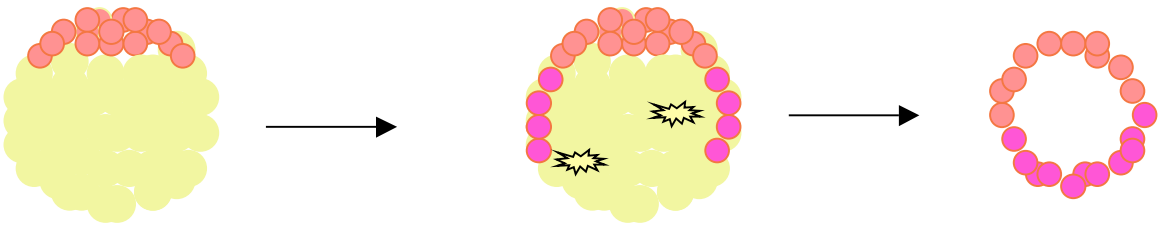
a.



b.



c.



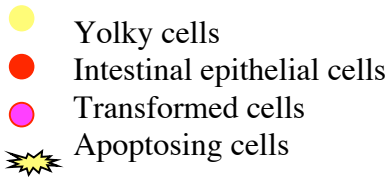
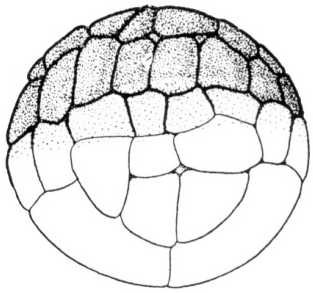
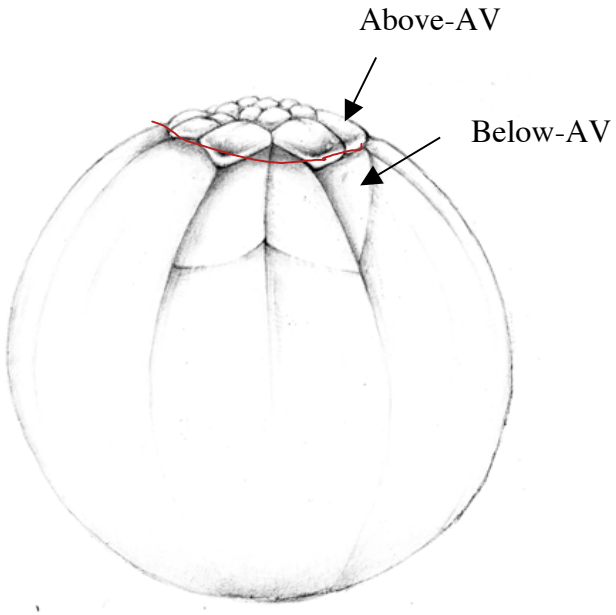

Yolky cells
Intestinal epithelial cells
Transformed cells
Apoptosing cells

Fig. 2: Comparison of *Xenopus* and *E. coqui* embryos.

(a) *Xenopus* embryo at 64-cell stage. All the blastomeres are completely divided and the animal blastomeres are pigmented (After Nieuwkoop and Faber, 1956). (b) *E. coqui* embryo at about 60-cell stage. Note the absence of pigmentation and the incompletely divided yolk rich vegetal blastomeres. The animal vegetal boundary: AV (-------) separates the animalmost small cells (Above-AV) from the larger vegetal cells (Below-AV).



(a)



(b)

Fig. 3: Vegetal pole injections: FDA injected in one of the vegetal blastomeres ended up only in the yolky endoderm (y) and not in the differentiated gut tissues (g).

Yolky endoderm (y) labeled in isolated guts of *E. coqui* frogs at various stages: (a) TS13, (b) TS15, (c) Newly hatched, (d) One week post-hatched (e) Bright fluorescent streaks in the yolky endoderm of a newly hatched *E. coqui*, (f) Control *E. coqui* gut (uninjected).

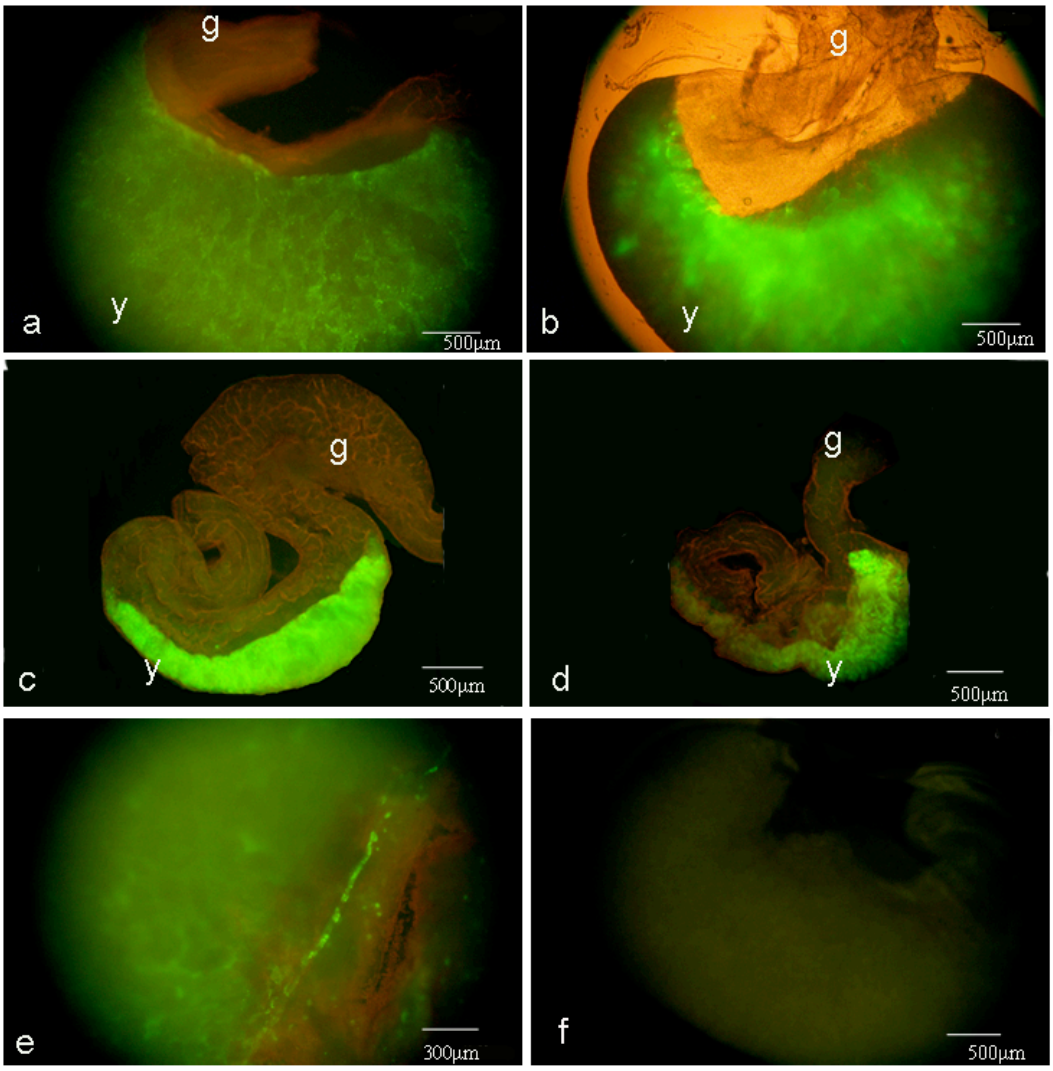


Fig. 3

Fig. 4: Vegetal pole injections: Most of the yolky endoderm in the developing gut is used up by one week post-hatched frogs. (a) Small amount of labeled yolky endoderm (y) left in the gut of one week post-hatched *E. coqui*. No label in foregut (fg), stomach (s) and hindgut (hg). (b) Appearance of label in the cloaca (c) and yolky endoderm (y) in a two weeks post-hatched stage *E. coqui*. No label in foregut (fg), and stomach (s). (c) Dull label in cloaca (c) and yolky endoderm (y) in a two week post-hatched *E. coqui*. Label in the cloaca was because of debris that oozed out after cloaca was punctured. Debris did not have any intact nuclei. (d) DAPI staining of debris from cloaca of an uninjected two week post-hatched stage *E. coqui* (e) DAPI staining of cloacal wall of the above frog.

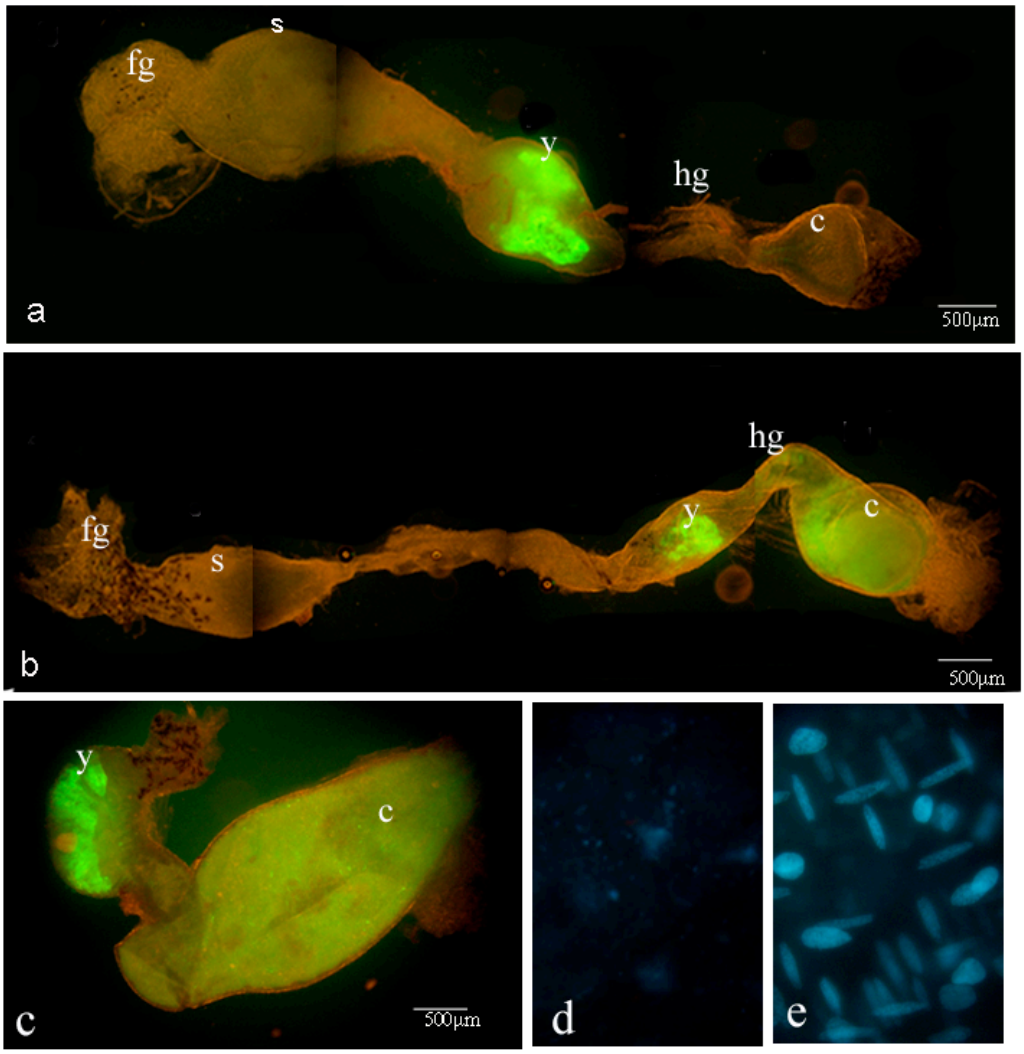


Fig. 4

Fig. 5: Vegetal pole injections: Apart from the yolky endoderm, vegetal pole FDA injections also labeled pronephros and mesonephros. Label in mesonephros first appeared weakly at TS13, and increased gradually in the later stages. Label in pronephros disappeared after TS15. (a) Confocal image of pronephros in a TS13 embryo. (b) Mesonephros of a TS15 *E. coqui*. (c) Mesonephros of a newly hatched *E. coqui*. (d) Control mesonephros (uninjected). (e) Confocal image of mesonephros of a newly hatched *E. coqui*. (f) Confocal image of two mesonephric tubules from the mesonephros in (e).

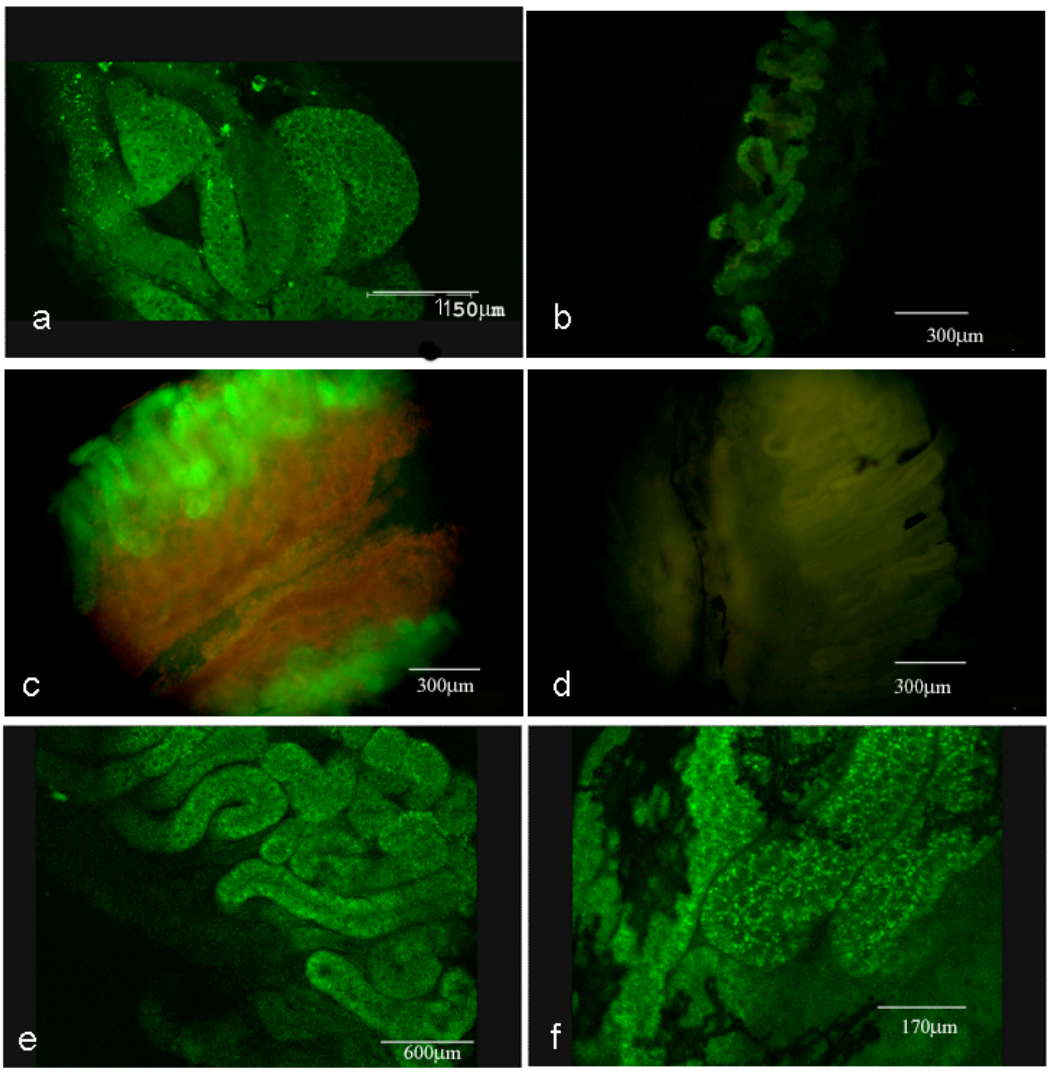


Fig. 5

Fig. 6: Vegetal pole injections. Almost all of the yolky endoderm is used up by the developing gut in one week post-hatched *E. coqui*. (a) Few yolky cells (y) left in the form of tiny specks (arrow) in the gut of a late one week post-hatched *E. coqui* (day 20). (b) Magnified image of the yolky cells in the above frog.

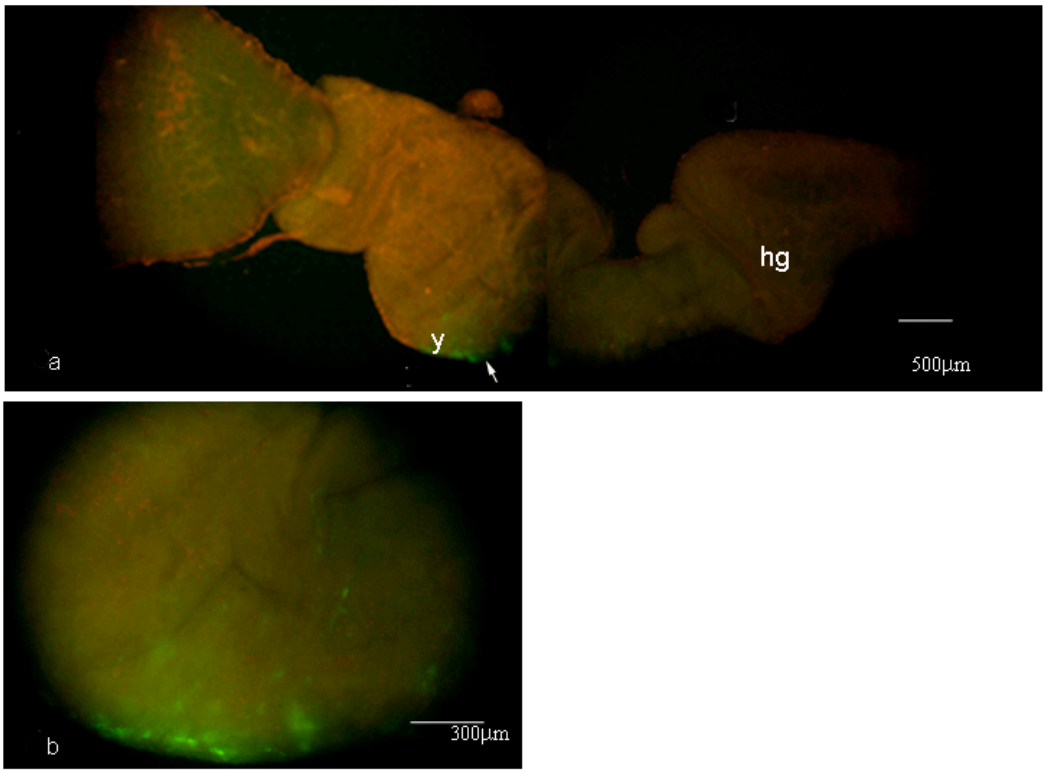


Fig. 6

Fig. 7: Contributions of above-AV blastomere: Above-AV FDA injections ended up in the organs coming from all the three germ layers: ectoderm, mesoderm and endoderm. (a) Labeling in the eye (arrows) of a TS15 *E. coqui*, (b) Confocal image of a half labeled spinal cord of a TS15 *E. coqui*, (c) Confocal image of a spinal cord labeled all the way along midline of a TS14 *E. coqui*, (d) Forelimb skin of a TS15 coqui, (e) Confocal image of mesonephros in a newly hatched *E. coqui*, (f) Confocal image of mesonephric tubules of a newly hatched *E. coqui*.

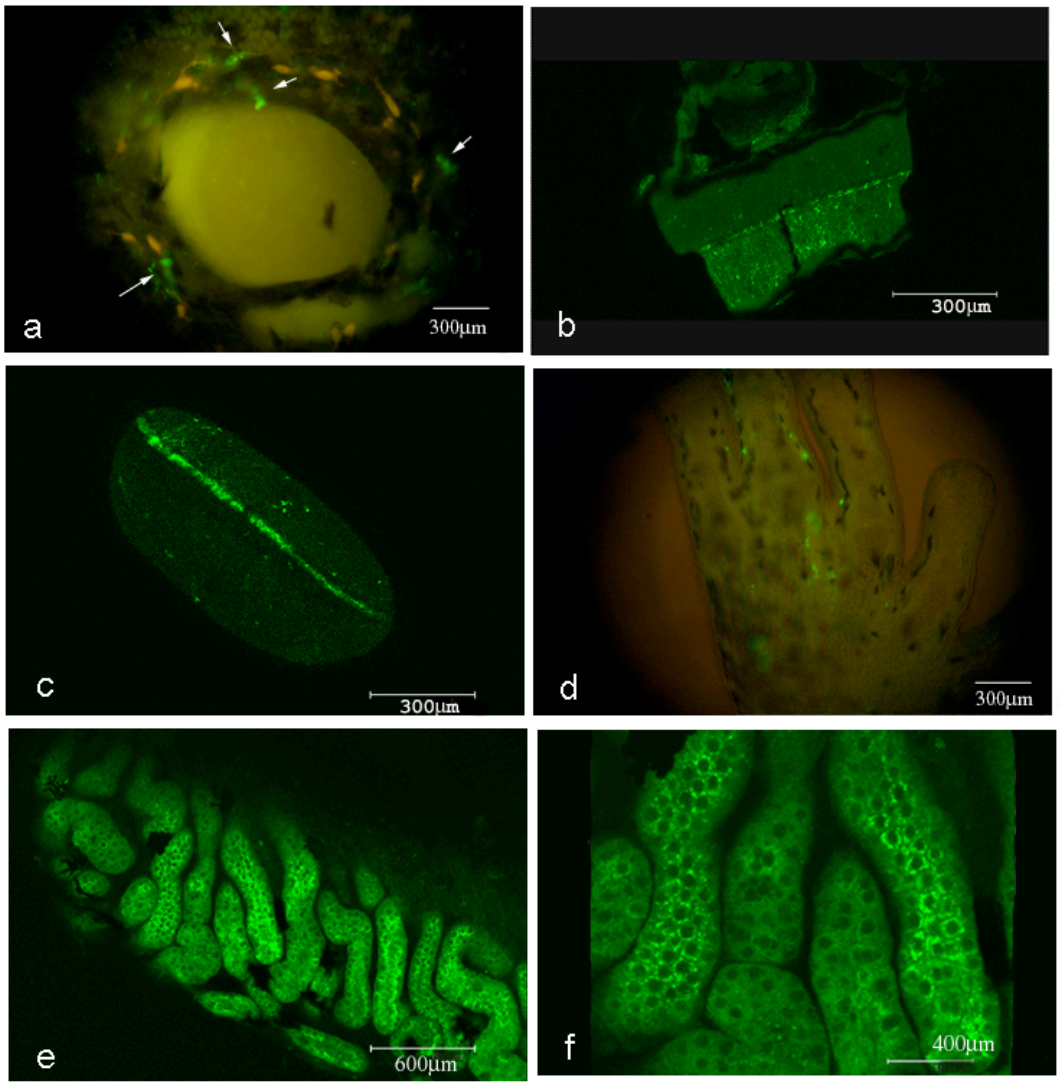


Fig. 7

Fig. 8: Contributions of above-AV blastomere:

(a) Hindlimb bone of a TS15 *E. coqui*, (b) Vertebrae of a TS15 *E. coqui*,

(c), (d) Fluorescent and phase contrast confocal images of a foregut in TS14 *E. coqui*.

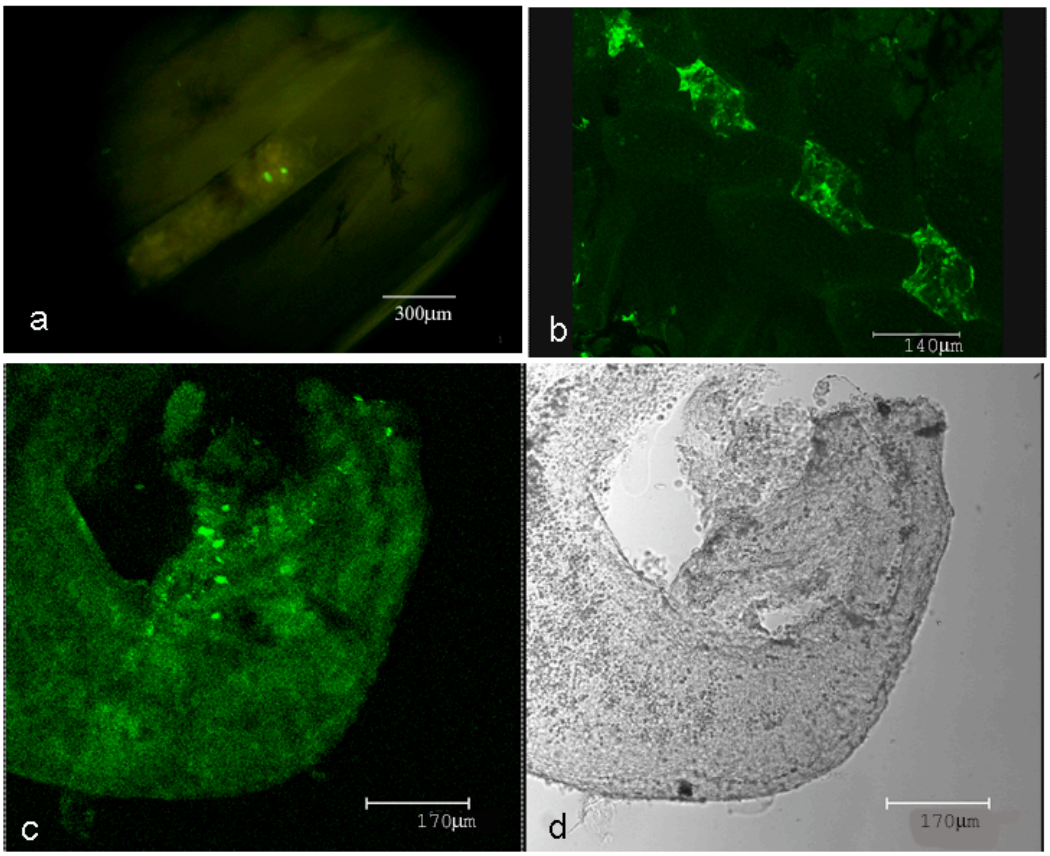


Fig. 8

Fig. 9: Below-AV injections: Below-AV blastomere also contributed to tissues coming from all three germ layers. (a) Confocal image of the start of the intestine of a TS15 *E. coqui*, (b) Overlap of confocal fluorescent and phase contrast images of the above intestine. Above-AV and below-AV injections labeled the yolky endoderm but showed distinct patterns. Label in the yolky endoderm from above-AV Injections was restricted to the edges (c) and in the interiors from below-AV injections (d).

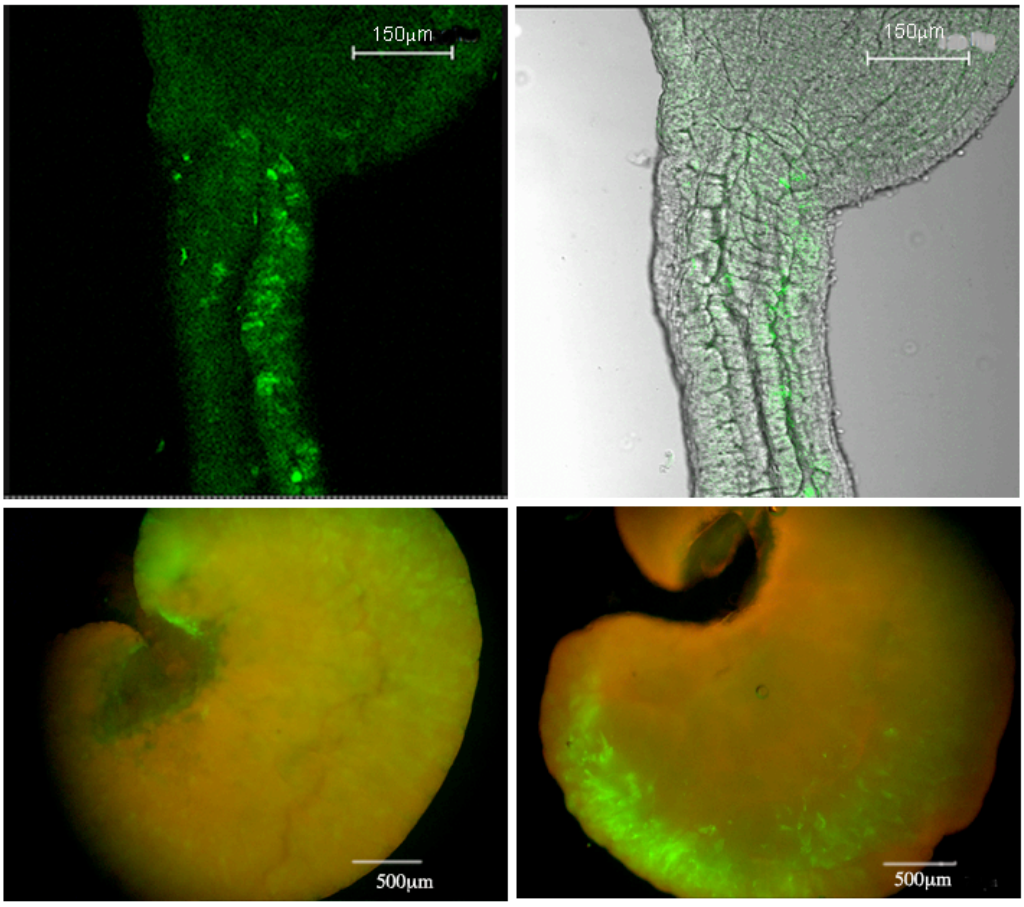


Fig. 9

Fig. 10. Mesonephric Kidneys
(a) Mesonephros) , (b) Nephron.

Redrawn from biology.Kenyon.edu/
& bill.srnr.Arizona.edu/classes/182/lecture-11.htm

(a) (b)

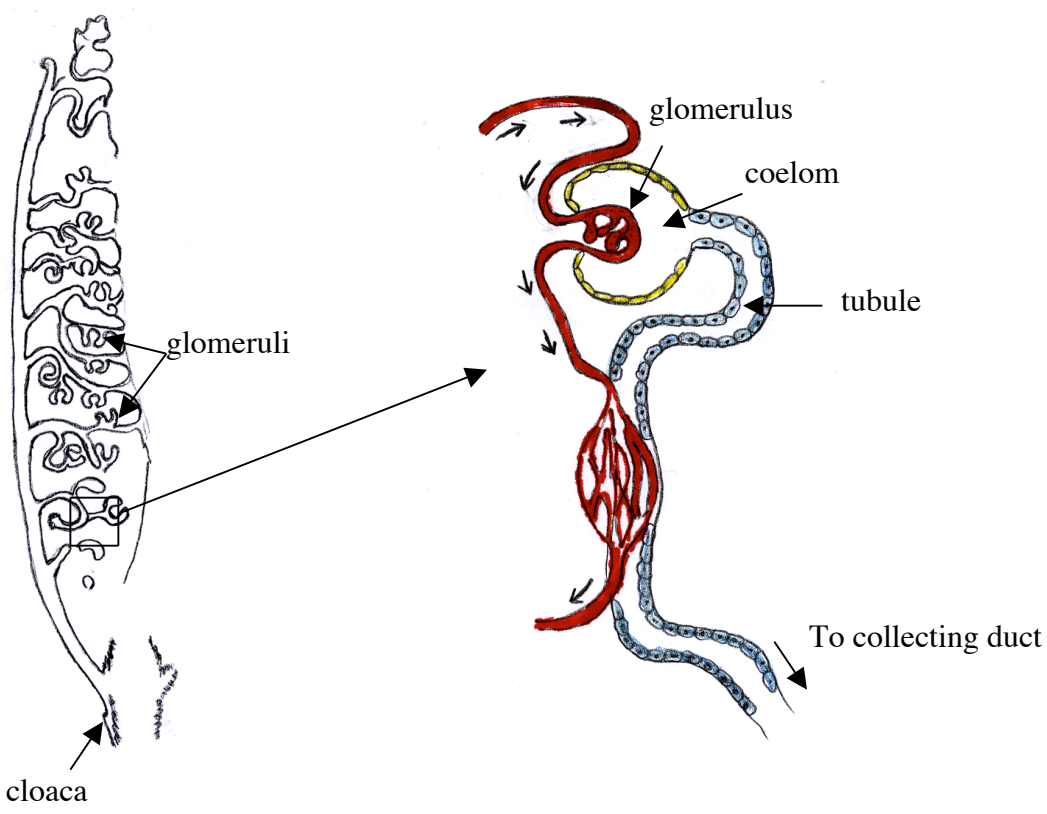

glomerulus
coelom
tubule
glomeruli
To collecting duct
cloaca

Table 1:Number of frogs scored for VP, Above AV and Below AV injections at various stages.

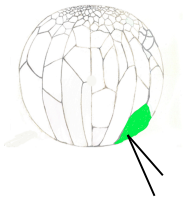
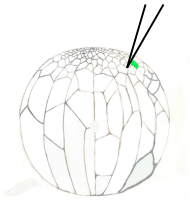
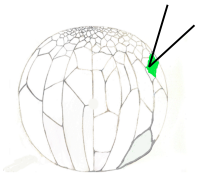
Injection	VP	Above AV	Below AV
			
	#	#	#
Stage	clutches / embryos	clutches / embryos	clutches / embryos
TS 9	2 / 2	? / 2	
TS 11	? / 2		
TS 12		? / 2	
TS 13	1 / 4		? / 11
TS 14		1 / 1	
TS 15	3 / 5	2 / 4	1 / 1
Newly hatched	6 / 13	1 / 3	2 / 6
1wk PH	10 / 15	2 / 3	? / 5
2 wk PH	5 / 8		
4 wk PH	1 / 1		
Total embryos	50	15	23

Table 2: Percent contributions of above-AV and below-AV blastomeres:
The percentages indicate the percentages of frogs having a label in the respective organs.

Blastomere injected	More than 90%	50-90%	10-50%	Less than 10%
Above-AV (n = 15)	Brain Nerve tube Yolky endoderm (periphery) Vertebrae	Eyes Liver mesonephros	Forelimbs Hind limbs Foregut Pronephros	Hindgut
Below-AV (n = 23)	Yolky endoderm mesonephros	Foregut	Brain Forelimbs Hind limbs Liver Hindgut Pronephros Nerve tube Vertebrae	Eyes

Table 3: Above-AV blastomere contributions: In an interesting group of embryos developing from above-AV injections, in a few embryos, the forelimbs and hind limbs were never labeled whenever endodermal organs like liver, gut tube and stomach were labeled. There were a few other that showed the exactly opposite condition namely label in hind and forelimbs but not in any part of the gut.

Forelimbs / Hindlimbs

Liver / Endoderm	+	–
+	7	5
–	3	-

REFERENCES

Arturson, G., Groth, T., Grotte, G. (1971). Human glomerular membrane porosity and filtration pressure: dextran clearance data analyzed by theoretical models. Clin Sci. 40, 37-58.

Beckham, Y. M., Nath, K., Elinson, R. P. (2003). Localization of RNAs in oocytes of *Eleutherodactylus coqui*, a direct developing frog, differs from *Xenopus laevis*. Evol Dev. 6, 562-571.

Berggard, I. (1970). Plasma proteins in normal human urine. In: Proteins in normal and pathological urine. Y. Manuel, J.P Revillard, and H. Betuel, editors. University Park Press, Baltimore, pp. 7-19.

Bott P.A Richards A.N. (1941). The passage of protein molecules through the glomerular membranes. J Biol Chem 141**,** 291-310.

Callery, E. M., Fang, H., Elinson, R. P. (2001). Frogs without polliwogs: evolution of anuran direct development. BioEssays. 23, 233-241.

Callery, E.M., Elinson, R.P. (2000a). Opercular development and ontogenetic re-organization in a direct developing frog. Dev Genes Evol. 210, 377-381.

Callery, E. M., Elinson, R. P. (2000b). Thyroid hormone-dependent metamorphosis in a direct developing frog. Proc Natl Acad Sci. 97, 2615-2620.

Casey, E.S., Tada, M., Fairclough, L., Wylie, C.C., Heasman, J., Smith, J.C. (1999). *Bix4* is activated directly by VegT and mediates endoderm formation in *Xenopus* development. Development. 126, 4193-4200.

Caulfield, J.P., Farquhar, M.G. (1974). The permeability of glomerular capillaries to graded dextrans. Identification of the basement membrane as the primary filtration barrier. J Cell Biol. 63, 883-903.

Chalmers, A. D., Slack, J. M. W. (1998). Development of the gut in *X. laevis*. Dev Dyn. 212, 509-21.

Chalmers, A.D., Slack, J.M. (2000). The *Xenopus* tadpole gut: fate maps and morphogenetic movements. Development. 127(2), 381-92.

Clements, D., Woodland, H. R. (2003). VegT induces endoderm by self-limiting mechanism and by changing the competence of cells to respond to TGF-β signals. Dev Biol. 258, 454-463.

Clements, D., Cameleyre, I., Woodland, H. R. (2003). Redundant early and overlapping larval roles of *XSox17* subgroup genes in *Xenopus* endoderm development. Mech Dev. 120, 337-348.

Clements, D., Friday, R. V., Woodland, H. R. (1999). Mode of action of VegT in mesoderm and endoderm formation. Development. 128, 4903-4911.

Dale, L., Slack, J. M. (1987). Regional specification within the mesoderm of early embryos of *Xenopus laevis*. Development. 100, 279-95.

Drummond, I.A., Majumdar, A., Hentschel,H., Elger, M., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Stemple, D.L., Zwartkruis, F., Rangini,Z., Driever, W., Fishman, M.C. (1998). Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. Development. 125, 4655-67.

Duellman, W.E., Trueb, L. (1986). Biology of Amphibians. McGraw-Hill Book Company. pp. 405-408.

Elinson, R. P. and Beckham, Y. (2002). Development in frogs with large eggs and the origin of amniotes. Zoology. 105, 105-117.

Elinson, R. P., Fang, H. (1998). Secondary coverage of the yolk by the body wall in the direct developing frog, *Eleutherodactylus coqui*: an unusual process for amphibian embryos. Dev Genes Evol. 208, 457-66.

Elinson, R. P. (1990). Direct development in frogs: Wiping the recapitulationist slate clean. Sem Dev Biol. 1, 263-270.

Elinson, R.P., Del Pino, E.M., Townsend,D.S., Cuesta, F.C., Eichhorn, P. (1990). A practical guide to the developmental biology of terrestrial-breeding frogs. Biol. Bull. 179, 163-177.

Engleka, M. J., Craig, E. J., Kessler, D. S. (2001). VegT activation of *Sox17* at the midblastula transition alters the response to nodal signals in the vegetal endoderm domain. Dev Biol. 237, 59-72.

Fang, H., Elinson, R. P. (1996). Patterns of distal-less gene expression and inductive interactions in the head of the direct developing frog *Eleutherodactylus coqui*. Dev Biol. 179, 160-172

Fang, H., Marikawa, Y., Elinson, R.P. (2000). Ectopic expression of Xenopus noggin RNA induces complete secondary body axes in embryos of the direct developing frog *Eleutherodactylus coqui*. Dev Genes Evol. 210, 21-7.

Graham, R.C.Jr., Karnovsky, M.J. (1966). Glomerular permeability. Ultrastructural cytochemical studies using peroxidases as protein tracers. J Exp Med.124, 1123-34.

Hardwicke, J. (1972). Glomerular filtration of macromolecules. Adv Nephrol Necker Hosp. 2,61-74.

Harland, R., Gerhart, J. (1997). Formation and function of Spemann's organizer. Ann Rev Cell Dev Biol. 13, 611-67

Heasman, J., Snape, A., Turner, A, Wylie CC. (1989). The establishment of regional identity in the *Xenopus* blastula. Ciba Found Symp. 144, 99-109.

Heasman J. (1997). Patterning the *Xenopus* blastula. Development. 124,4179-91

Henry, G. L., Melton, D. A. (1998). Mixer, a homeobox gene required for endoderm development. Science. 281, 91-96.

Holowacz, T., Elinson, RP. (1993). Cortical cytoplasm, which induces dorsal axis formation in *Xenopus*, is inactivated by UV irradiation of the oocyte. Development 119, 277-85.

Horb, M. E., Thomsen, G. H. (1997). A vegetally localized T-box transcription factor in *Xenopus* eggs specifies mesoderm and endoderm and is essential for embryonic mesoderm formation. Development. 124, 1689-98

Horb, M. E., Slack, J. M. (2001). Endoderm specification and differentiation in *Xenopus* embryos. Dev Biol. 236, 330-43.

Hudson, C., Clements, D., Friday, R. V., Stott, D., Woodland, H. R. (1997). *Xsox17alpha* and *-beta* mediate endoderm formation in *Xenopus*. Cell. 91, 397-405.

Hyatt, B.A., Yost, H.J. (1998). The left-right coordinator: The role of Vg1 in organizing left-right axis formation. Cell. 93, 37-46.

James, J.A., Ashworth, C.T. (1961). Some features of glomerular filtration and permeability revealed by electron microscopy after intraperitonial injection of dextran in rats. Am. J. Pathol. 38, 515-525.

Karnovsky MJ, Ainsworth SK. (1972). The structural basis of glomerular filtration. Adv Nephrol Necker Hosp. 2, 35-60.

Karnovsky, M.J. (1979). The ultrastructure of glomerular filtration. Ann Rev Med. 30, 213-24.

Keller, R.E. (1975). Vital dye mapping of the gastrula and neurula of Xenopus laevis. I. Prospective areas and morphogenetic movements of the superficial layer. Dev Biol. 42, 222-41.

Kimelman, D., Christian, J.L., Moon, R.T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. Development.116, 1-9.

Kessler DS, Melton DA (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. Development 121, 2155-64.

Kofron, M., Demel, T., Xanthos, J., Lohr, J., Sun, B., Sive, H., Osada, S., Wright, C., Wylie, C., Heasman, J. (1999). Mesoderm induction in *Xenopus* is a zygotic event regulated by maternal VegT via TGFβ growth factors. Development. 126, 5759-5770

Langer, C.E. (2003). Uncoiling the gut of *Eleutherodactylus coqui*: Characterization of anatomical development and proliferation pattern. M.S thesis, Duquesne University.

Lee, S. (2005). Kidney development in Eleutherodactylus coqui with relation to edema syndrome. M.S thesis, Duquesne University

Marikawa, Y., Elinson, RP. (1998). β-TrCP is a negative regulator of Wnt/beta-catenin signaling pathway and dorsal axis formation in *Xenopus* embryos. Mech Dev. 77,75-80.

Melton, D.A. (1987). Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. Nature. 328, 80-2.

Mogensen, C.E. (1968). The glomerular permeability determined by dextran clearance using Sephadex gel filtration. Scand J Clin Lab Invest. 21,77-82.

Nieuwkoop, P.D., and J. Faber, ed. 1956. Normal Table of *Xenopus laevis* (Daudin): A Systematical and Chronological Survey of the Development from the Fertilized Egg till the End of Metamorphosis. North-Holland Publishing Co, Amsterdam.

Ninomiya, H., Zhang, Q., Elinson, RP. (2001). Mesoderm formation in *Eleutherodactylus coqui*: body patterning in a frog with a large egg. Dev Biol. 236, 109-23.

Renkin, E.M., Robinson, R.R. (1974). Glomerular filtration. N Engl J Med. 290, 785-92.

Renkin, E.M., and Gilmore, J.P. 1973. Glomerular filtration. Handb. Physiol (Sect.8 Renal Physiol.): 185-248

Singamsetty, S. (2005). Expression of the endodermal marker, *EcSox17*, during embryogenesis of the direct developing frog, *Eleutherodactylus coqui*. M.S thesis, Duquesne University.

Sinner, D., Rankin, S., Lee, M., Zorn, A. M. (2004). *Sox17* and _-*catenin* cooperate to regulate the transcription of endodermal genes. Development. 131, 3069-3080.

Stennard, F., Carnac, G., Gurdon, J.B. (1996). The *Xenopus* T-box gene, *Antipodean*, encodes a vegetally localized maternal mRNA and can trigger mesoderm formation. Development. 122, 4179-4188.

Tada, M., Casey, E. S., Fairclough, L., Smith, J.C. (1998). Bix 1, a direct target of *Xenopus* T-box genes, causes formation of ventral mesoderm and endoderm. Development. 125, 3997-4006.

Thomsen, G.H., Melton, D.A. (1993). Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. Cell. 74, 433-41.

Townsend, D. S., Stewart, M. M. (1985). Direct development in *Eleutherodactylus coqui* (Anura: Leptodactylidae): a staging table. Copeia 1985, 423-436

Tortora, G.J., Derrickson, B. (2006). Principles of Anatomy and Physiology. John Wiley & Sons. pp. 992-1006.

Venkatachalam, M.A., Karnovsky, M.J., Fahimi, H.D., Cotran, R.S.(1970) An ultrastructural study of glomerular permeability using catalase and peroxidase as tracer proteins. J Exp Med. 132,1153-67

Wallenius, G. (1954). Renal clearance of Dextran as a measure of glomerular permeability. Acta Soc Med Ups Suppl. 59, 1-99.

Weeks, D. L., Melton, D.A. (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGFβ. Cell. 51. 861- 7.

White, R. J., Sun, B. I., Sive, H. L., Smith, J. C. (2002). Direct and indirect regulation of derrière, a *Xenopus* mesoderm-inducing factor, by VegT. Development. 129, 4867-4876.

Xanthos, J.B., Kofron, M., Wylie, C., Heasman, J. (2001). Maternal VegT is the initiator of a molecular network specifying endoderm in *Xenopus laevis*. Development. 128, 167-180.

Yasuo, H., Lemaire, P. (1999). A two-step model for the fate determination of presumptive endodermal blastomeres in *Xenopus* embryos. Curr Biol. 9, 869-879.

Yuge, M., Kobayakawa, Y., Fujisue, M., Yamana, K. (1990). A cytoplasmic determinant for dorsal axis formation in an early embryo of Xenopus laevis. Development. 110, 1051-6.

Zhang, J., King, M.L. (1996). *Xenopus* VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. Development. 122, 4119-4129.